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PRINCIPAL INVESTIGATOR: Derek Sieburth

CONTRACTING ORGANIZATION: University of Colorado
Boulder, Colorado 80309

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**6. AUTHOR(S)**
Derek Sieburth

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Colorado
Boulder, Colorado 80309

**e-mail:**
derek.sieburth@colorado.edu

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**13. ABSTRACT** *(Maximum 200 Words)*
The goal of this research is to understand the regulation of Ras-mediated signaling in *C. elegans* vulval development. We describe the identification and characterization of a novel gene, *sur-8*, that functions to regulate a receptor tyrosine kinase-Ras-MAP kinase-mediated signal transduction pathway during *C. elegans* vulval development. Mutations in *sur-8* were identified as suppressors of an activated *let-60 ras* mutation. Our genetic analysis indicates that *sur-8* plays a positive regulatory role in Ras-mediated signaling, and appears to function downstream of Ras but not downstream of Raf. Although *sur-8* mutations by themselves have no effect on normal Ras-mediated signaling, reduction of *sur-8* function dramatically enhances *mpk-1* MAP kinase and *ksr-1* mutations and an increase of *sur-8* dosage enhances an activated *let-60 ras* mutation. We found that *sur-8* encodes a novel protein conserved in mammals that is composed predominantly of leucine-rich repeats. SUR-8 interacts directly with LET-60 Ras, but fails to interact with a putative effector domain mutant, P34G. A structural and functional SUR-8 homologue in humans specifically binds K-Ras and N-Ras but not H-Ras in vitro. Our results indicate that *sur-8* is an evolutionarily conserved positive regulator of Ras signaling pathways and that SUR-8 may mediate its effects through Ras binding. We also describe evidence that a regulatory B subunit of Protein Phosphatase 2A (PP2A) positively regulates an RTK-Ras-MAP kinase signaling cascade during *Caenorhabditis elegans* vulval induction. Although reduction of *sur-6* PP2A-B function causes few vulval induction defects in an otherwise wild-type background, *sur-6* PP2A-B mutations suppress the Multivulva phenotype of an activated *ras* mutation and enhance the Vulvaless phenotype of mutations in *lin-45 raf, sur-8* or *mpk-1*. Double mutant analysis suggests that *sur-6* PP2A-B acts downstream or in parallel to *ras* but likely upstream of *raf,* and functions with *ksr-1* in a common pathway to positively regulate Ras signaling.

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ANNUAL REPORT For Predoctoral Fellowship
Award # DAMD17-96-1-6117

**"Genetic and Molecular Analysis of Suppressors of Ras Mutations"**

## INTRODUCTION:

The Ras family of proteins play critical roles in cell proliferation, differentiation and migration in response to extracellular signals. The Han lab is interested in studying the regulation of the Ras mediated signal transduction pathway in the nematode *C. elegans*. The Ras pathway controls vulval development in *C. elegans*, and the purpose of this work has been to use sensitive genetic screens to identify components of the Ras signal transduction pathway. This approach has led to the discovery of several genes that act downstream of ras as well as several regulatory genes that modify the strength of signaling. The work funded by this grant has led to the discovery of two additional regulators of the Ras pathway signaling, *sur-8* and *sur-6*. *sur-8* plays a positive regulatory role in Ras-mediated signaling, and appears to function downstream of Ras but not downstream of Raf. Although *sur-8* mutations by themselves have no effect on normal Ras-mediated signaling, reduction of *sur-8* function dramatically enhances *mpk-1* MAP kinase and *ksr-1* mutations and an increase of *sur-8* dosage enhances an activated *let-60 ras* mutation. *sur-8* encodes a novel protein conserved in mammals that is composed predominantly of leucine-rich repeats. SUR-8 interacts directly with LET-60 Ras, but fails to interact with a putative effector domain mutant, P34G. A structural and functional SUR-8 homologue in humans specifically binds K-Ras and N-Ras but not H-Ras in vitro. Our results indicate that *sur-8* is an evolutionarily conserved positive regulator of Ras signaling pathways and that SUR-8 may mediate its effects through Ras binding. *sur-6* encodes a regulatory B subunit of Protein Phosphatase 2A (PP2A) and also positively regulates an RTK-Ras-MAP kinase signaling cascade during *Caenorhabditis elegans* vulval induction. Although reduction of *sur-6* PP2A-B function causes few vulval induction defects in an otherwise wild-type background, *sur-6* PP2A-B mutations suppress the Multivulva phenotype of an activated *ras* mutation and enhance the Vulvaless phenotype of mutations in *lin-45 raf, sur-8* or *mpk-1*. Double mutant analysis suggests that *sur-6* PP2A-B acts downstream or in parallel to *ras* but likely upstream of *raf,* and functions with *ksr-1* in a common pathway to positively regulate Ras signaling.

## BODY: Annual Summary

The research on *sur-6* and *sur-8* has been published. The results sections are printed here. The work on *sur-8* was reported in last years annual summary report.

### SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans*

To identify factors that act downstream of Ras during vulval induction, we screened for extragenic suppressor mutations that would revert the Multivulva (Muv) phenotype caused by a gain-of-function *let-60 ras* mutation, *n1046gf*, back to wild type.

The *n1046gf* mutation encodes a Gly to Glu substitution at codon 13 (G13E) (Beitel et al., 1990) also found in human Ras oncoproteins (Bos, 1988). By screening for suppressors of this activated *ras* mutation, we expected to identify mutations in genes acting downstream of or in parallel to *let-60 ras* or in genes involved in the proper expression or activation of *let-60 ras*. The parental strain used for screening carries multiple copies of a *let-60 ras(n1046gf)* genomic fragment (Sundaram et al., 1996) and displays a completely penetrant Muv phenotype. This increased penetrance of the Muv phenotype over that caused by non-transgenic *let-60 ras(n1046gf)* animals allowed us to rapidly screen a large number of genomes for suppressor mutations. From 22,000 haploid genomes screened, we isolated 11 mutations in at least four genes, including a single mutation in the *sur-8* locus (suppresor of *ras*), *ku167*, three alleles of *lin-45 raf* and 3 alleles of *mek-2* MEK. We identified a second allele of *sur-8, ku242*, in a non-complementation screen that was not biased against isolating null mutations. *sur-8(ku242)* failed to complement the suppression phenotype of *sur-8(ku167)* in a *let-60 ras(n1046gf)* background.

Both *sur-8(ku167)* and *sur-8(ku242)* mutations suppressed the Muv phenotype caused by *let-60 ras(n1046gf)* to nearly wild type and suppressed the male mating defect associated with *let-60 ras(n1046gf)*. For example, the *sur-8(ku167)* mutation reduced the Muv phenotype of *let-60 ras(n1046gf)* animals from 87% to 4% (Table 1). The suppression observed was due to a decrease in the average vulval induction of the VPCs from 154% to 102% (Table 1). Both *sur-8* mutations most often reverted the pattern of ectopic vulval induction back to a wild type pattern (data not shown). Since *sur-8(ku167)* was a slightly stronger suppressor than *sur-8(ku242)*, further genetic characterization was performed using *sur-8(ku167)*.

Genetic dosage analysis indicated that the *sur-8(ku167)* mutation is a recessive, strong loss-of-function mutation. The deficiency *mDf4* failed to complement *sur-8(ku167)* for the Suppression phenotype. Animals in which *ku167* was in trans to *mDf4*, and thus contained only one copy of *sur-8(ku167)*, displayed a suppression phenotype that was similar to, but slightly stronger than, animals homozygous for *sur-8(ku167)* (Table 1). In addition, the duplication *mDp1*, which covers the *sur-8* locus, reverted the suppression phenotype of *sur-8(ku167) let-60(n1046gf)* animals to 90% Muv (Table 1). Finally, a mutant *sur-8(ku167)* gene when overexpressed in *sur-8(ku167) let-60(n1046gf)* mutants retained very little but some *sur-8* activity (data not shown). Thus, the *sur-8(ku167)* mutation results in severe reduction but probably not elimination of *sur-8(+)* function.

Table 1. Phenotype and Gene Dosage Analysis of *sur-8* Mutant Animals

| # | *sur-8* genotype[a] | *let-60 ras* genotype | phenotype %Muv(n)[b] | phenotype % induction (n)[c] |
|---|---|---|---|---|
| 1 | + | + | 0 (many) | 100 (many) |
| 2 | + | *gf* | 87 (276) | 154 (27) |
| 3 | *ku167/ku167* | *gf* | 4 (333) | 102 (43) |
| 4 | *ku167/+* | *gf* | 77 (57) | 129 (16) |
| 5 | *ku242/ku242* | *gf* | 7 (328) | 103 (33) |

| | | | | |
|---|---|---|---|---|
| 6 | *ku242/+* | *gf* | 84 (175) | 153 (15) |
| 7 | *ku167/ku242* | *gf* | 11 (160) | 103 (31) |
| 8 | *ku167/ku167; mDp1* | *gf* | 90 (200) | 154 (32) |
| 9 | *+; mDp1* | *gf* | 100 (247) | 182 (30) |
| 10 | *+; kuEx83* | *gf* | 100 (200) | 196 (18) |
| 11 | + | *gf/+* | nd | 106 (30) |
| 12 | *+; kuEx83* | *gf/+* | nd | 145 (28) |
| 13 | *ku167/mDf4*[d] | *gf* | 14 (270) | 108 (32) |
| 14 | *+/mDf4*[d] | *gf* | 68 (233) | 136 (22) |

Although *sur-8(ku167)* and *sur-8(ku242)* suppressed the Muv phenotype caused by the activated *let-60 ras(n1046gf)* mutation, neither allele caused an apparent phenotype in a *let-60 ras(+)* background. Mutants displayed wild type vulval induction (Table 2) and appeared to have no additional obvious developmental defects (data not shown). However, the positive role that *sur-8* plays in vulval induction became apparent when examining its effects on Ras-mediated signaling in sensitised genetic backgrounds. First, as described above, *sur-8* mutations could strongly suppress both the Muv and male mating defects caused by the *let-60 ras(n1046gf)* allele. Second, an extrachromosomal array containing multiple copies of the cloned *sur-8* gene enhanced the average vulval induction of both *let-60(n1046gf) /let-60(n1046gf)* homozygous animals (from 154% to 196%) and *let-60(n1046gf) /+* heterozygous animals (Table 1).

Finally, *sur-8 (ku167)* severely affected vulval induction when other *ras* pathway components were compromised. *sur-8(ku167)* dramatically enhanced Vulvaless and larval lethal phenotypes caused by a weak loss-of-function mutation in *mpk-1* (Wu and Han, 1994). *mpk-1(ku1)* mutants alone display nearly wild type vulval induction and only 7% rod-like larval lethality, but *sur-8(ku167)* decreased vulval induction to 0% and increased larval lethality to nearly 100% in the double mutants (Table 2). Because *mpk-1* MAP kinase is a component of the main Ras pathway, this observed genetic interaction suggests that *sur-8* is an important positive regulator of the Ras pathway that functions to increase pathway output. Furthermore, *sur-8(ku167)* also showed strong genetic interactions with a loss-of-function mutation in another regulator of the Ras pathway, *ksr-1* (Sundaram and Han, 1995). *ksr-1(ku68)* mutants alone display wild type vulval induction (100%) and a weak rod-like lethal phenotype (24%). In *sur-8(ku167); ksr-1(ku68)* double mutants, vulval induction was reduced to 4% and the rod-like larval lethality was increased to 85% (Table 2). This strong genetic interaction between *sur-8* and *ksr-1* suggests that while the function of neither gene is normally required for Ras signaling, their functions are collectively essential for Ras signaling.

Table 2. Genetic Interactions between *sur-8* and *ksr-1* or *mpk-1* MAP kinase Mutations

| genotype | %Induction[a] P3.p P4.p P5.p P6.p P7.p P8.p | %Induction (n) | %Lethal[b] (n |
|---|---|---|---|

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| N2 (wild type) | 0 | 0 | 100 | 100 | 100 | 0 | 100 | (many) | 0 (138) |
| *sur-8(ku167)* | 0 | 0 | 100 | 100 | 100 | 0 | 100 | (28) | 0 (244) |
| *sur-8(ku242)* | 0 | 0 | 100 | 100 | 100 | 0 | 100 | ( ) | 0 (347) |
| *mpk-1(ku1)*[c] | | | | | | | 98 | (17) | 7 (229) |
| *mpk-1(ku1) sur-8(ku167)*[c] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | (25) | 100 (80) |
| *ksr-1(ku68)*[d] | 0 | 0 | 100 | 100 | 100 | 0 | 100 | (15) | 24 (257) |
| *sur-8(ku167); ksr-1(ku68)*[d] | 0 | 0 | 0 | 11 | 0 | 0 | 4 | (19) | 85 (164) |

To determine at which step in the linear Ras pathway *sur-8* may function, we performed epistasis analysis with mutations that cause Muv phenotypes. *sur-8* mutations almost completely suppress the Muv phenotype caused by *let-60 ras(n1046gf)*, suggesting that *sur-8* acts downstream of or in parallel to *let-60 ras* (Table 3). We tested whether *sur-8* mutations could suppress the Muv phenotype caused by a loss-of-function mutation in *lin-15, n765*. *lin-15* functions upstream of *ras* at the level of *let-23* RTK to inhibit *let-23* signaling (Ferguson et al., 1987) and encodes two novel proteins that are expressed in the surrounding hypodermis (Clark et al., 1994; Huang et al., 1994). As expected, *sur-8* mutations could suppress the *lin-15* mutant Muv phenotype (Table 3), consistent with *sur-8* functioning downstream of *let-60 ras*. However, this suppression was not complete, possibly due to the inability of *sur-8* mutations to overcome strong pathway activity caused by the *lin-15(n765)* mutation.

In order to define a downstream limit for *sur-8* function, we tested whether a *sur-8* mutation could suppress the Muv phenotype caused by genes acting downstream of *ras*. *lin-1* is a negative regulator of the Ras pathway and encodes a putative transcription factor that by genetic criteria acts downstream of *mpk-1* MAP kinase (Beitel, et al., 1995; Wu et al., 1994; Kornfeld et al., 1995). A loss-of-function mutation of *lin-1, ar147*, causes a 100% Muv phenotype that was not suppressed by *sur-8(ku167)*. Double mutants displayed the same 100% Muv phenotype as the *lin-1* mutants (Table 3), suggesting that *sur-8* does not act downstream and probably acts upstream of *lin-1*.

Genetic studies indicate that *lin-45 raf* acts downstream of *let-60 ras* in the vulval induction pathway (Han et al., 1993). Animals carrying an activated *raf(gf)* transgene under the control of a heat shock promoter displayed a Muv phenotype upon heat shock (Table 3). As expected, the Muv phenotype was completely suppressed by weak mutations in either *mek*-2 or *mpk-1* (Table 3), which act downstream of *lin-45 raf* (Kornfeld, 1997; Sundaram and Han, 1996). However, a *sur-8* mutation failed to suppress the Muv phenotype caused by the *raf(gf)* transgene. Heat shocked *raf(gf)* mutants or *sur-8(ku167); raf(gf)* double mutants displayed similar average vulval induction of 116% and 125%, respectively (Table 3), indicating that *sur-8* does not function downstream of *lin-45 raf* in the same linear pathway as *mek-2* and *mpk-1*. Taken together, the epistasis data indicate that *sur-8* acts downstream or in parallel to *let-60 ras* but not downstream of *lin-45 raf*.

Table 3. Epistatic Analysis of *sur-8(ku167)* and Muv Mutants

| genotype[a] | | %Muv (n) | | %Induction (n) | |
|---|---|---|---|---|---|
| + | *let-60(n1046)* | 88 | (240) | 154 | (27) |

| | | | | |
|---|---|---|---|---|
| *sur-8(ku167) let-60(n1046)* | 4 | (333) | 102 | (43) |
| + ; *HSP-raf(gf)* | 36 | (31) | 116 | (31) |
| *sur-8(ku167); HSP-raf(gf)* | 64 | (28) | 125 | (28) |
| *mek-2(ku114); HSP-raf(gf)* | 0 | (25) | 97 | (25) |
| *mpk-1(ku1); HSP-raf(gf)* | 0 | (28) | 99 | (28) |
| + ; *lin-15 (n765)* | 98 | (214) | 190 | (24) |
| *sur-8(ku167); lin-15 (n765)* | 73 | (302) | 142 | (21) |
| *sur-8(ku242); lin-15 (n765)* | 58 | (258) | 133 | (23) |
| *lin-1(ar147)* + | 100 | (154) | ND | |
| *lin-1(ar147) sur-8(ku167)* | 100 | (184) | ND | |

Vulval cell fate specification takes place at the end of the L2 stage, after the anchor cell is born and before the VPCs undergo their first division (Kimble, 1981). To determine if *sur-8(+)* activity is required at this stage for proper vulval induction, we assayed the ability of *sur-8(+)* to rescue the suppression phenotype of *sur-8(ku167) let-60(n1046gf)* animals at various stages of development. We generated transgenic *sur-8(ku167) let-60(n1046gf)* animals carrying *sur-8* cDNA (see below) under the control of a heat inducible promoter and subjected them to heat shock at different developmental stages. Control transgenic animals without heat shock displayed a slightly rescued phenotype of 123% vulval induction probably resulting from leaky *sur-8* expression from the heat shock promoter. Animals heat shocked before or during vulval induction (between early L2 and mid L3 stages) displayed a fully rescued phenotype resulting in over 160% induction (Table 4) similar to that observed in *sur-8(ku167) let-60(n1046gf)* mutants carrying a transgene of *sur-8* under control of its own promoter (Table 1). In contrast, animals heat shocked either in L1, before the anchor cell is born, or in L4, after Pn.p cells have executed their fate, displayed only a partially rescued phenotype of 133% or 134% vulval induction (Table 4). The rescuing activity observed in early L2 heat shocked animals is most likely due to SUR-8 protein perdurance. Thus, *sur-8(+)* activity is required before or during the time of vulval cell fate specification for vulval development but is not required at earlier or later times.

Table 4. Recsue of *sur-8(ku167) let-60(n1046)* Phenotype by Human *sur-8* cDNA under Control of a Heat Shock Promoter

| extrachromosomal | induction % | | | | | | total | |
|---|---|---|---|---|---|---|---|---|
| array | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | induction | n |
| vector | 0 | 8 | 100 | 100 | 100 | 6 | 103% | 38 |
| Ce *sur-8* | 61 | 97 | 100 | 100 | 100 | 93 | 184% | 42 |
| Hs *sur-8* | 16 | 40 | 100 | 100 | 100 | 70 | 142% | 25 |

We cloned *sur-8* by genetic mapping followed by transformation-rescue (see Experimental Procedures). *sur-8* was mapped to position 1.86 on chromosome IV between the markers *dpy-13* and *unc-5*. Cosmids containing genomic DNA from this region were tested for *sur-8(+)* activity by assaying their ability to revert the Suppressed

phenotype of *sur-8(ku167) let-60(n1046gf)* animals back to Muv. A single cosmid, AC7, contained complete rescuing activity as did a 12kb AC7-derived subclone (data not shown). The subclone was predicted to contain a single gene, designated AC7.1 by the *C. elegans* genome sequencing project. A full length 2.1 kb cDNA was identified by screening a mixed stage library (gift from Peter Okkema) using a genomic probe derived from the predicted AC7.1 gene. Northern blot analysis indicated that this cDNA was the only transcript encoded by *sur-8* (data not shown). We conclude that the gene defined by the isolated cDNA corresponds to *sur-8* because missense mutations from *sur-8* mutant DNA are located in the coding region of the cDNA, and because this cDNA was able to rescue *sur-8* mutants (see below).

The 1.7 kb coding sequence of *sur-8* is predicted to encode a novel 559 amino acid protein containing 18 tandem repeats of the leucine-rich repeat (LRR) motif (amino acids 89-505) (Figure 2). LRRs, characterized by a consensus composed of leucines at invariant positions, are found in a variety of proteins with diverse biological functions and are proposed to mediate protein-protein interactions (Kobe and Deisenhofer, 1994). 15 of the 18 SUR-8 LRRs are 23 amino acids long and form a consensus that is similar to that of yeast adenylate cyclase LRRs (Figure 3). These 23 amino acid LRRs form two tandem clusters of 9 and 6 repeats that are separated by 3 tandem LRRs that are 24 amino acids long and form a distinct consensus with no obvious similarity to other known LRR motifs. SUR-8 contains N-terminal and C-terminal non-LRR flanking sequences of 88 and 53 amino acids, respectively (Figure 2).

We have identified a missense mutation associated with each *sur-8* allele (Figure 2). *sur-8(ku242)* encodes a cysteine 233 to tyrosine substitution in a consensus position within LRR 7. *sur-8(ku167)* encodes a glutamic acid 430 to lysine substitution in a non-consensus position within LRR 15. Both mutations were found to alter amino acids conserved in mammalian *sur-8* homologues (Figure 2 and see below) indicating that these residues may have an evolutionarily conserved function.

An expressed sequence tag (EST) database search revealed several overlapping human and mouse ESTs that shared from 49% to 70% amino acid identity with the non-LRR C-terminal sequences of Ce SUR-8. We used primers derived from a human or mouse EST (GenBank accession numbers W51818 and AA286839, respectively) to amplify the 5' ends of the cDNAs by performing 5' RACE from human brain cDNA or mouse liver cDNA (Clonetech). Sequences from the 5' RACE and EST clones were compiled to generate the full-length (4.1 kb) human and mouse *sur-8* cDNAs. Multi-tissue Northern blot (Clonetech) analysis using a probe derived from the human cDNA revealed that this cDNA corresponded to a single transcript of the predicted size, and the transcript was detected in all tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (data not shown). The predicted proteins encoded by the human and mouse cDNAs are 98% identical at the amino acid level.

Comparison of the amino acid sequences encoded by the mammalian and *C. elegans sur-8* genes revealed significant homology in amino acid sequence and overall protein structure. Mammalian SUR-8 contains exactly the same number of LRRs with the identical length and organization as *C. elegans* SUR-8 LRRs (Figures 2 and 3). The LRR regions share 58% identity and the C-terminal extensions share 76% identity while the N-terminal extensions share very little identity (Figure 2). The 23 amino acid repeats

in Hs SUR-8 have the same consensus sequence as their Ce SUR-8 counterparts (Figure 3).

We found that in addition to sharing a high degree of structural homology, the *C. elegans* and human SUR-8 proteins share functional homology. Human *sur-8* cDNA expressed under the control of a heat shock inducible promoter was able to rescue the mutant phenotype of *sur-8(ku167) let-60(n1046gf)* animals (Table 4). Hs *sur*-8 could revert the Suppressed phenotype from 103% induction to 142% induction. Control animals expressing Ce *sur-8* cDNA displayed a fully rescued phenotype with 184% induction (Table 4). Because Hs *sur-8* could provide *sur-8(+)* activity in *sur-8* mutants, we conclude that Hs *sur-8* is a functional homologue of Ce *sur-8*.

Yeast adenylate cyclase contains 26 LRRs (Kataoka et al., 1985) which are required for binding to and activation by Ras during vegetative yeast growth (Field et al., 1990; Suzuki et al., 1990). The observation that SUR-8 LRRs form a consensus that is similar to that of yeast adenylate cyclase LRRs led us to speculate that SUR-8 may bind LET-60 Ras. We used the yeast two-hybrid system to test the interaction between SUR-8 and several Ras pathway components. While we failed to detect an interaction between SUR-8 and wild type LIN-45 Raf, MEK-2 MEK, MPK-1 MAP kinase or KSR-1 (data not shown), we detected an interaction with wild type LET-60 Ras, as assayed by the activation of a His reporter (Figures 4 and 5A) and a lacZ reporter (data not shown).

Given that mutations in the LRR regions result in loss of *sur-8* function, we examined the effect of these mutations on LET-60 RAS interaction. Interestingly, while *ku167* E430K had no effect on LET-60 Ras interaction, *ku242* C233Y eliminated detectable interaction with LET-60 Ras (Figure 4A), even though both mutant proteins were expressed at similar levels in yeast (data not shown). The *ku242* C233Y mutation is in LRR 7 of the N-terminal LRR cluster and these data demonstrate that this residue is critical for both SUR-8 function and LET-60 Ras binding.

To define a specific region of SUR-8 involved in LET-60 Ras binding, we tested SUR-8 deletion mutants for LET-60 Ras interaction (Figure 4A). Deletion of the N-terminal 88 amino acid non-LRR region resulted in elimination of LET-60 Ras binding. Similarly, deletion of the C-terminal 53 amino acid non-LRR sequence resulted in reduction of LET-60 Ras binding. In addition, deletion of the last six LRRs, including repeat 15, abolished LET-60 Ras binding. It is likely that N-or C-terminal deletions alter global protein structure that may result in decreased LET-60 Ras interaction.

We next wanted to define a domain of LET-60 Ras required for SUR-8 interaction. We tested interaction of SUR-8(+), and as a control, LIN-45 Raf, with several different LET-60 Ras mutants using the yeast two-hybrid system. Mutations tested included point mutations that cause phenotypes in *C. elegans* (Beitel et al., 1990), deletion mutations, and effector domain mutations (Figure 4B). Point mutations in the effector domain of H-Ras have been shown to abolish binding to several putative Ras binding proteins, including Raf1, PI-3 kinase, and Ral-GDS (Rodriguez-Viciana et al., 1997).

All of the LET-60 Ras loss-of-function mutations tested had no effect on SUR-8 or LIN-45 Raf binding. In addition, the mutation encoded by the gain-of-function allele, *n1046gf* G13E, had no effect on SUR-8 binding or LIN-45 Raf binding. Deletion of the membrane targeting region had only a slight effect on SUR-8 binding or LIN-45 Raf binding. An effector domain double mutation, E37G Y40C, interfered with LIN-45 Raf

binding, but had no effect on SUR-8 binding (Figure 4B), suggesting that SUR-8 does not share binding specificity with Raf.

However, we identified one effector domain mutation, P34G, that specifically interfered with SUR-8(+) binding but had no effect on LIN-45 Raf binding. A P34G Ras mutant has previously been shown to bind Raf1 with wild type affinity in vitro, but fails to have transforming activity or neurite outgrowth-inducing activity in vivo (Akasaka et al., 1996). SUR-8 is thus a likely candidate for promoting full Ras activity through binding the Ras effector domain at a site that is distinct from that of Raf.

Given the functional and structural homology between Ce *sur-8* and Hs *sur-8*, we were interested in determining whether Hs SUR-8 could bind mammalian Ras. We tested the interaction of Hs SUR-8 with three human Ras family members, N-Ras, K-Ras 4B and H-Ras, in the yeast two-hybrid system. We detected a strong interaction between Hs SUR-8 and two family members, K-Ras and N-Ras but detected only a weak interaction with H-Ras (Figure 5A). As a control for Ras expression, we showed that all three Ras family members interacted strongly with Raf1 (Figure 5A). In addition SUR-8 and Ras displayed cross-species interactions, reinforcing the idea that *sur-8* function may be evolutionarily conserved.

These observations were confirmed by testing in vitro interaction of Hs SUR-8 and Ras family members (Figure 5B). Bacterially expressed GST-Ras fusion proteins were purified and tested for their ability to interact with purified Hs SUR-8 or Raf1. While all three family members bound Raf1 with similar affinities, only N-Ras and K-Ras were capable of binding Hs SUR-8 strongly. However, while Raf1 bound Ras with GTP dependence, Hs SUR-8 showed no GTP dependence for Ras binding. Both GDP and GTP loaded Ras proteins bound Hs SUR-8 with similar affinities. Thus, SUR-8 displayed a differential binding specificity for individual Ras family members that appeared not to depend on the activation state of Ras.

**A PP2A regulatory subunit positively regulates Ras-mediated signaling during *C. elegans* vulval induction**

We screened for mutations that suppress the Multivulva phenotype caused by an activated *let-60 ras* mutation, *n1046gf* (e.g. Sundaram and Han 1995), or that enhance the Vulvaless phenotype caused by a hypomorphic *lin-45 raf* mutation, *ku112* (M.S., unpublished). These screens identified two mutations, *ku123* and *cs24*, that define the gene *sur-6* (suppressor of *ras*). These *sur-6* mutations cause few or no vulval defects in an otherwise wild-type background; *ku123* and *cs24* mutants display an average vulval induction of 100% or 99%, respectively (Table 1). However, both *sur-6* mutations suppress the Multivulva phenotype of *let-60(n1046gf)* animals (Table 2), and enhance the vulval induction defects and larval lethality caused by weak alleles of *lin-45 raf* (Table 1). These strong genetic interactions suggest that the *sur-6* mutations reduce signaling by the Ras pathway at a point downstream or in parallel to *let-60 ras*.

*sur-6(ku123)* and *sur-6(cs24)* appear to strongly reduce (but not eliminate) *sur-6* gene function. These *sur-6* alleles and a deficiency of the *sur-6* locus each semi-dominantly suppress the Multivulva phenotype of *let-60(n1046gf)* mutants (Table 2), suggesting the *sur-6* locus is haplo-insufficient. RNA-mediated inhibition of *sur-6* also suppresses the *let-60(n1046gf)* Multivulva phenotype and causes a partial Vulvaless phenotype in a wild-type background (Table 3), arguing that these are loss-of-function phenotypes.

*qDf8* fails to complement the weak Vulvaless phenotype of *sur-6(cs24)* mutants, and *sur-6/sur-6* homozygotes and *sur-6/qDf8* hemizygotes display similar phenotypes (Tables 1,2), consistent with the *sur-6* mutations strongly reducing *sur-6* function. This notion is further supported by the fact that the suppressor phenotype of *sur-6(ku123)* can be rescued by injecting wild-type DNA containing the *sur-6* gene (see below and Materials and Methods). Nevertheless, the *sur-6* mutations are likely non-null, since RNA inhibition suggests that the *sur-6* null phenotype is embryonic lethal (Table 3; see below). Since reducing *sur-6* function reduces vulval induction in sensitized genetic backgrounds, we conclude that *sur-6* normally plays a positive role in regulating Ras pathway signaling during vulval development.

Table 1. *sur-6* mutations enhance the vulval defects of *lin-45* Raf, *mpk-1* MAP kinase, or *sur-8* mutants, but not *ksr-1* mutants

| Genotype[a] | Vul[b] | Average Induction[c] | (n) | Larval Lethal[d] (n) |
|---|---|---|---|---|
| *sur-6(ku123)* | 0% | 100% | (26) | 0% (474) |
| *sur-6(ku123)/qDf8* | 0% | 100% | (28) | 0% (531)[e] |
| *sur-6(cs24)* | 2% | 99% | (48) | 0% (195) |
| *sur-6(cs24)/qDf8* | 8% | 97% | (26) | 0% (386)[e] |
| *+/qDf8* | 0% | 100% | (16) | nd |
| *cs24/+* | 0% | 100% | (35) | nd |
| *lin-45(sy96)* | 58% | 53% | (38) | 86% (290) |
| *sur-6(ku123); lin-45(sy96)* | 100% | 5% | (17) | 94% (228) |
| *lin-45(ku112)* | 0% | 100% | (26) | <1% (66) |
| *sur-6(cs24); lin-45(ku112)* | 87% | 50% | (24) | 80% (360) |
| *mpk-1(ku1)* | 17% | 97% | (29) | 7% (229) |
| *sur-6(ku123); mpk-1(ku1)* | 82% | 76% | (28) | 77% (263) |
| *sur-8(ku167)* | 0% | 100% | (18) | <1% (271) |
| *sur-6(ku123); sur-8(ku167)*[f] | 71% | 56% | (24) | <1% (263) |
| *sur-6(cs24); sur-8(ku167)* | 65% | 67% | (20) | 3% (198) |
| *ksr-1(ku68)* | 0% | 100% | (23) | 24% (257) |
| *sur-6(ku123); ksr-1(ku68)* | 3% | 99% | (40) | 17% (282) |
| *sur-8(ku167); ksr-1(ku68)*[g] | 100% | 4% | (19) | 85% (164) |
| *ksr-1(n2526)* | 1% | 99% | (68) | 2% (607) |
| *sur-6(cs24); ksr-1(n2526)* | 3% | 99% | (62) | 1% (88) |
| *sur-8(ku167); ksr-1(n2526)* | 67% | 64% | (24) | 54% (132) |

*sur-8* and *ksr-1* are two other genes that display genetic interactions similar to those of *sur-6*. Strong loss-of-function mutations in *sur-8* or *ksr-1* cause few defects on their

own, but strongly modify the phenotypes of other Ras pathway mutants (Kornfeld et al. 1995; Sundaram and Han 1995; Sieburth et al. 1998). We found that *sur-6;sur-8* double mutants display a synthetic Vulvaless phenotype (Table 1), consistent with these mutations potentially affecting different aspects of Ras pathway regulation. Interestingly, however, *sur-6; ksr-1* double mutants resemble the *sur-6* or *ksr-1* single mutants (Table 1). Because reducing the function of either *ksr-1* or *sur-6* has the same effect as reducing the function of both, it is likely that *sur-6* acts together with *ksr-1* in a common signaling pathway to regulate Ras signaling.

Table 2. Epistasis analysis of *sur-6* and *ksr-1* with Multivulva mutations.

| *sur-6* or *ksr-1* mutation[a] | Muv mutation[b] | Multivulva[c] (n) | Average Induction[d] (n) |
|---|---|---|---|
| +/+ | +/+ | 0% (many) | 100% (many) |
| +/+ | *let-60(n1046gf)* | 87% (276) | 154% (27) |
| *sur-6(ku123)* | *let-60(n1046gf)* | 6% (240) | 103% (26) |
| *sur-6(cs24)* | *let-60(n1046gf)* | 19% (107) | 104% (33) |
| *sur-6(ku123)/sur-6(cs24)* | *let-60(n1046gf)* | 3% (31) | nd |
| *sur-6(ku123)/+* | *let-60(n1046gf)* | 22% (148) | 115% (31) |
| *sur-6(cs24)/+* | *let-60(n1046gf)* | 40% (53) | nd |
| *sur-6(ku123)/qDf8* | *let-60(n1046gf)* | 1% (209) | nd |
| *+/qDf8* | *let-60(n1046gf)* | 5% (281) | nd |
| +/+ | *HSP-raf(gf)* | 47% (30) | 119% (30) |
| *sur-6(ku123)* | *HSP-raf(gf)* | 47% (17) | 125% (17) |
| +/+ | *HSP-raf(gf)* | 47% (17) | 118% (17) |
| *ksr-1(ku68)* | *HSP-raf(gf)* | 46% (28) | 117% (28) |
| +/+ | *lin-1(e1275)* | 99% (68) | nd |
| *sur-6(ku123)* | *lin-1(e1275)* | 100% (102) | nd |
| +/+ | *lin-15(n765)* | 100% (59) | 157% (28) |
| *sur-6(ku123)* | *lin-15(n765)* | 6% (351) | 102% (20) |

*raf* genetically acts downstream of *ras* in *C. elegans* and in *Drosophila* (Dickson et al. 1992; Han et al. 1993), and mammalian Raf is a direct Ras effector (reviewed by Katz and McCormick 1997). *Drosophila ksr* has been shown previously to function genetically downstream or in parallel to *ras* and upstream of *raf* (Therrien et al. 1995). We tested whether this was also true for *C. elegans ksr-1* and *sur-6*. An activated *raf* transgene under control of a heat shock promoter [*HSP-raf(gf)*]causes a Muv phenotype which can be suppressed by mutations in the downstream Ras pathway components *mek-2* MEK or *mpk-1* MAP kinase (Sieburth et al. 1998). In contrast, this *HSP-raf(gf)* Multivulva

phenotype is not suppressed by *ksr-1* or *sur-6* mutations (Table 2). Therefore, *ksr-1* and *sur-6* likely act upstream of *lin-45 raf*. Consistent with this, *sur-6(ku123)* also fails to suppress the Multivulva phenotype caused by a relatively weak mutation of *lin-1*, which encodes an ETS domain transcription factor acting downstream of *mpk-1* MAP kinase (Jacobs et al. 1998; Tan et al. 1998). The genetic placement of *ksr-1* and *sur-6* suggests that *ksr-1* and *sur-6* may act in a regulatory branch that modifies or cooperates with Ras/Raf.

We cloned *sur-6* by genetic mapping followed by transformation rescue (see Materials and Methods). An 11 kb fragment, pDS89, which contains *sur-6*(+) rescuing activity was predicted by the *C. elegans* genome sequencing consortium to contain a single gene (F26E4.1) that encodes a PR55 family regulatory B subunit of protein phosphatase 2A (PP2A-B, Fig. 1). The *sur-6* alleles *ku123* and *cs24* each contain single G to A substitutions that introduce amino acid substitutions at highly conserved positions within PP2A-B (Fig. 1). The PP2A-B coding region, when expressed under the control of a heat shock promoter in transgenic animals, can rescue the *sur-6(ku123)* mutant (see Materials and Methods), and RNA interference of PP2A-B phenocopies the *sur-6* suppression and partial Vulvaless phenotypes (Table 3). Thus we conclude that *sur-6* encodes PP2A-B. SUR-6 shares over 59% overall amino acid identity with PP2A-B from human or *Drosophila,* with three large stretches of at least 75% identity (Fig. 1B). In mammals there are three PR55/B isoforms that differ in spatial and temporal expression (Mayer-Jaekel and Hemmings 1994). SUR-6 is most similar to the mammalian Bα subtype.

Table 3. RNA interference of *sur-6* PP2A-B, PP2A-C or PP2A-A

| Genotype | dsRNA[a] or transgenes[b] | Vul or Muv (n) | Avg. Induction (n) |
|---|---|---|---|
| + | no dsRNA | 0% (many) | 100% (many) |
| + | *sur-6* PP2A-B | 19% Vul (21) | 94% (21) |
| + | PP2A-C | 5% Vul (22) | 99% (22) |
| *let-60(n1046gf)* | no dsRNA | 95% Muv (19) | 173% (19) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 3% Muv (29) | 101% (29) |
| *let-60(n1046gf)* | PP2A-C | 70% Muv (23) | 132% (23) |
| *let-60(n1046gf)* | *sur-8* | 15% Muv (33) | 106% (33) |
| *let-60(n1046gf)* | no dsRNA | 93% Muv (59) | 143% (59) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 7% Muv (15) | 103% (15) |
| *let-60(n1046gf)* | PP2A-A | 57% Muv (14) | 125% (14) |
| *let-60(n1046gf)* | no transgenes | 82% Muv (242) | 175% (23) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 20% Muv (172) | 110% (48) |

PP2A-B subunits modulate the activity and/or substrate specificity of the PP2A-A/C catalytic core (Mayer-Jaekel and Hemmings 1994). SUR-6 is the only predicted PR55/B family member encoded by the *C. elegans* genome, although other types of regulatory B

subunits (such as PR56/B') are also present. The *C. elegans* genome is predicted to encode a single PP2A-A subunit (F48E8.5) and a single PP2A-C subunit (F38H4.9), which share over 90% amino acid identity with their mammalian counterparts. As expected, the PP2A-A subunit can bind to both SUR-6 PP2A-B and the PP2A-C subunit, as assayed by the yeast two-hybrid system (data not shown). Given the positive role of *sur-6* PP2A-B defined by genetic analysis, *sur-6* PP2A-B could either function to activate the catalytic core, which in turn would activate Ras pathway signaling, or it could function to relieve inhibition of Ras signaling by the core complex (Fig. 2A).

To determine the requirements for PP2A during *C. elegans* development, we used RNA interference (RNAi) (Fire et al. 1998) to block *sur-6* PP2A-B, PP2A-A or PP2A-C expression. For each PP2A gene (but not for *sur-8* or *ksr-1*), RNAi caused highly penetrant embryonic lethality in both wild-type and *let-60(n1046gf)* backgrounds. Embryos arrested at about the 100 cell stage, with widely variable cell sizes (data not shown). Thus, unlike *ksr-1* and *sur-8*, PP2A appears to be absolutely required during embryonic development in addition to functioning later during vulval induction. Since the *sur-6(ku123)* and *sur-6(cs24)* mutations caused little or no embryonic lethality, even when hemizygous (Table 2), the two functions of *sur-6* PP2A-B appear separable, with these *sur-6* point mutations primarily affecting *sur-6* PP2A-B function in vulval development but not in embryogenesis.

To avoid the PP2A(RNAi) lethality and test its effects on vulval development, we examined the last surviving progeny of RNA injected mothers (Table 3). In this assay, *sur-6* RNAi caused a partial Vulvaless phenotype in a wild-type background and efficiently suppressed the *let-60(n1046gf)* Multivulva phenotype. However, PP2A-C(RNAi) caused few vulval defects and only weakly suppressed the *let-60(n1046gf)* Multivulva phenotype. Similar results were obtained by using a hypodermal-specific promoter to drive expression of *sur-6* PP2A-B or PP2A-C RNAs (Table 3). Thus it is still unclear whether PP2A catalytic activity promotes and/or inhibits Ras-mediated vulval induction.

## APENDIX TO SUMMARY:

### 1) Key research accomplishments:

- Identification of *sur-8* as a positive regulator of Ras signaling.
- Demonstration that *sur-8* acts at the level of Ras or Raf.
- Cloning of *sur-8*, which encodes a novel leucine-rich repeat protein.
- Demonstration that SUR-8 interacts with Ras proteins.
- Identification of Mammalian homologues of SUR-8, and demonstration of functional homology in *C. elegans*.
- Identification of *sur-6* as a positive regulator of Ras signaling.
- Cloning of *sur-6* which encodes a regulatory subunit of protein phosphatase 2A.
- Demonstration that SUR-6 acts with KSR-1 to regulate Ras signaling upstream of Raf.

### 2) Reportable outcomes:

**Publications from this work:**

SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans*. Sieburth et al., Cell 94:119-130 (1998).

A PP2A regulatory subunit positively regulates Ras-mediated signaling during *C. elegans* vulval induction. Sieburth et al., Genes and Development. in press. (1999).

**Platform presentations:**

May 1998 — West Coast Worm Meeting (Berkeley, CA)
"SUR-8: a conserved Ras-binding protein with leucine-rich repeats positively regulates Ras-mediated signaling." D. Sieburth, Q. Sun and M. Han.

May 1997 — 11th International *C. elegans* Meeting (Madison, WI)
"*sur-6* and *sur-8* positively regulate Ras signaling in *C. elegans*." D. Sieburth and M. Han.

July 1996 — West Coast Regional *C. elegans* Meeting (Vancouver, BC)
"Genetic Analysis of *sur-6* an activator of the Ras pathway during vulval development." D. Sieburth and M. Han.

**Poster presentations:**

Oct. 1997 — The Department of Defense Breast Cancer Research Program Meeting (Washington, DC)
"*sur-6* and *sur-8* positively regulate Ras signaling in *C. elegans*." D. Sieburth and Min Han.

June 1995 — 10th International *C. elegans* Meeting (Madison, WI)
Poster: "*sur-4*: A negative regulator of the Ras pathway in vulval development." D. Sieburth, M. Hara and M. Han.

**Patents applied for:**
SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans.* Derek Sieburth and Min Han.

**Degrees obtained:**
PhD awarded to Derek Sieburth from the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder.

**Employment received:**
Derek Sieburth was awarded a postdoctoral fellowship from the Walter Winchell-Damon Runyon Cancer Research Fund for research done in the laboratory of Dr. Joshua M. Kaplan in the department of Molecular and Cellular Biology at the University of California, Berkeley.

**3) See enclosed manuscripts**

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# SUR-8, a Conserved Ras-Binding Protein with Leucine-Rich Repeats, Positively Regulates Ras-Mediated Signaling in *C. elegans*

**Derek S. Sieburth, Qun Sun, and Min Han***
Howard Hughes Medical Institute
Department of Molecular, Cellular
and Developmental Biology
University of Colorado at Boulder
Boulder, Colorado 80309-0347

## Summary

**We describe the identification and characterization of a novel gene, *sur-8*, that positively regulates Ras-mediated signal transduction during *C. elegans* vulval development. Reduction of *sur-8* function suppresses an activated *ras* mutation and dramatically enhances phenotypes of *mpk-1* MAP kinase and *ksr-1* mutations, while increase of *sur-8* dosage enhances an activated *ras* mutation. *sur-8* appears to act downstream of or in parallel to *ras* but upstream of *raf*. *sur-8* encodes a conserved protein that is composed predominantly of leucine-rich repeats. The SUR-8 protein interacts directly with Ras but not with the Ras(P34G) mutant protein, suggesting that SUR-8 may mediate its effects through Ras binding. A structural and functional SUR-8 homolog in humans specifically binds K-Ras and N-Ras but not H-Ras in vitro.**

## Introduction

The Ras family of proteins plays critical roles in cell proliferation, differentiation, and migration in response to extracellular signals. Biochemical studies using mammalian tissue culture and genetic analysis of *C. elegans* and *Drosophila* suggest that RTK-Ras-MAP kinase signal transduction pathway is not a simple linear pathway but is likely part of a complicated network (Wassarman et al., 1995; Sundaram and Han, 1996; Katz and McCormick, 1997). Two important questions remain to be addressed regarding the relationship between the linear Ras pathway and other factors involved in the signaling process. The first question is related to how activation of this pathway leads to diverse cellular responses. For example, if the Ras pathway is to be regulated at the level of Ras, the same Ras protein may have different upstream regulators as well as different downstream effectors for different functions. In recent years, several potential Ras effectors in addition to Raf have been described, including phosphatidylinositol-3-OH (PI-3) kinase and Ral GDS (Katz and McCormick, 1997), and are good candidates for defining branch points for Ras signaling. In addition, different Ras family members may perform distinct cellular functions by associating with unique sets of regulators or effectors.

In mammals, the Ras family is composed of four highly related members: H-Ras, N-Ras, K-Ras 4A, and K-Ras 4B (Barbacid, 1987). Family members are 100% identical in the first 86 amino acids, which contain the effector domain (amino acids 32–40), and are most divergent in the C-terminal 26 amino acids, which contain the lipid modified membrane-targeting domains (amino acids 164–189). K-*ras*, N-*ras*, and H-*ras* have widely overlapping spatial and temporal patterns of expression (Furth et al., 1987; Leon et al., 1987), and each family member is found mutated in certain tumor types (Bos, 1988), suggesting that they have overlapping functions. This notion is supported by studies showing that N-*ras* or H-*ras* deficient mice have no apparent abnormalities (Umanoff et al., 1995; M. Katsuki, unpublished, cited in Johnson et al., 1997). However, K-*ras* knockout mice exhibit embryonic lethality and early hematopoetic defects, phenotypes that are exacerbated by reduction of N-*ras* dosage (Johnson et al., 1997), providing evidence for a unique function for K-*ras*. Further support for distinct Ras functions comes from the observation that many tumor types are associated with activating mutations of primarily one particular Ras family member (Leon et al., 1987). It is thus also possible that different Ras family members interact with distinct subsets of proteins that mediate unique regulatory or effector functions.

The second question regarding the complexity of the Ras-mediated signaling processes is what collaborative roles multiple factors and signaling branches may have in regulating the output of the signal. The main components of the RTK-Ras-MAP kinase pathway may be essential elements of a given signaling process, but there may be other factors that feed into or out of this pathway that play important regulatory functions to ensure the maximal activity of the pathway and to tighten the regulation of the signal. For example, the *ksr* genes were identified as suppressors of activated *ras* in *C. elegans* and *Drosophila* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995), and their biochemical relationship with the Ras pathway is an interesting topic of research.

*C. elegans* provides a powerful genetic system to identify and characterize genes that regulate the Ras-mediated signal transduction pathway. In *C. elegans*, there is a single known *ras* gene, *let-60*, that acts in an RTK-Ras-MAP kinase signal transduction pathway to specify several cell fates, including those of the vulva (Figure 1). Many genes in the pathway function in multiple signaling events during *C. elegans* development. For example, *let-60 ras* has also been shown to function in male tail fate specification (Chamberlin and Sternberg, 1994), germ cell and oocyte development (Church et al., 1995; Gutch et al., 1998), sex-myoblast migration (Sundaram et al., 1996), and excretory duct cell fate specification (Yochem et al., 1997).

To identify new factors acting downstream of *let-60 ras*, our laboratory and others have screened for mutations that can suppress activated *let-60 ras* mutations (Sundaram and Han, 1996; Kornfeld, 1997). In addition to identifying genes that act in the main pathway downstream of *let-60 ras*, we have identified mutations in a number of new genes that can suppress the Multivulva

*To whom correspondence should be addressed.

(Muv) phenotype caused by activated *let-60 ras* but do not cause an obvious vulval phenotype on their own. Here we describe the identification and characterization of one such gene, *sur-8*. Our genetic analysis indicates that *sur-8* is likely to act downstream or parallel of *let-60 ras*, but upstream of *raf*, to positively regulate the signaling. We further show that *sur-8* encodes a novel but conserved protein that is predominantly composed of leucine-rich repeats (LRRs). Finally, we demonstrate that SUR-8 interacts with LET-60 Ras and that a highly conserved human SUR-8 homolog appears to have binding specificity for K-Ras and N-Ras but not H-Ras.

## Results

### Mutations in *sur-8* Suppress Activated *let-60 ras*

To identify factors that act downstream of Ras during vulval induction, we screened for extragenic suppressor mutations that would revert the Muv phenotype caused by a gain-of-function *let-60 ras* mutation, *n1046gf* or *G13E* (Beitel et al., 1990), back to wild type. The *G13E* allele has also been found in human Ras oncoproteins (Bos, 1988). For this study, we modified the previous screen by using a parental strain that carries multiple copies of a *let-60 ras(n1046gf)* genomic fragment (Sundaram et al., 1996) and displays a completely penetrant Muv phenotype. This increased penetrance of the Muv phenotype over that caused by nontransgenic *let-60 ras(n1046gf)* animals allowed us to rapidly screen a large number of genomes for suppressor mutations. From 22,000 haploid genomes screened, we isolated 11 mutations in at least four genes, including a single mutation in the *sur-8* locus (*suppressor of ras*), *ku167*, three alleles of *lin-45 raf*, and three alleles of *mek-2* MEK. We identified a second allele of *sur-8, ku242*, in a noncomplementation screen that was not biased against isolating null mutations. *sur-8(ku242)* failed to complement the suppression phenotype of *sur-8(ku167)* in a *let-60 ras (n1046gf)* background.

Both *sur-8(ku167)* and *sur-8(ku242)* mutations suppressed the Muv phenotype caused by *let-60 ras(n1046gf)* to nearly wild type and suppressed the male mating defect associated with *let-60 ras(n1046gf)*. For example, the *sur-8(ku167)* mutation reduced the Muv phenotype of *let-60 ras(n1046gf)* animals from 87% to 4% (Table 1). The suppression observed was due to a decrease in the average vulval induction of the VPCs from 154% to 102% (Table 1). Both *sur-8* mutations most often reverted the pattern of ectopic vulval induction back to a wild-type pattern (data not shown). Since *sur-8(ku167)* was a slightly stronger suppressor than *sur-8(ku242)*, further genetic characterization was performed using *sur-8(ku167)*.

Genetic dosage analysis indicated that the *sur-8(ku167)* mutation is a recessive, strong loss-of-function mutation. The deficiency *mDf4* failed to complement *sur-8(ku167)* for the suppression phenotype. Animals in which *ku167* was in *trans* to *mDf4*, and thus contained only one copy of *sur-8(ku167)*, displayed a suppression phenotype that was similar to, but slightly stronger than, animals homozygous for *sur-8(ku167)* (Table 1). In addition, the duplication *mDp1*, which covers the *sur-8* locus, reverted the suppression phenotype of *sur-8 (ku167) let-60(n1046gf)* animals to 90% Muv (Table 1). Finally, a mutant *sur-8(ku167)* gene when overexpressed in *sur-8(ku167) let-60(n1046gf)* mutants retained very little, but some, *sur-8* activity (data not shown). Thus, the *sur-8(ku167)* mutation results in severe reduction but probably not elimination of *sur-8(+)* function.

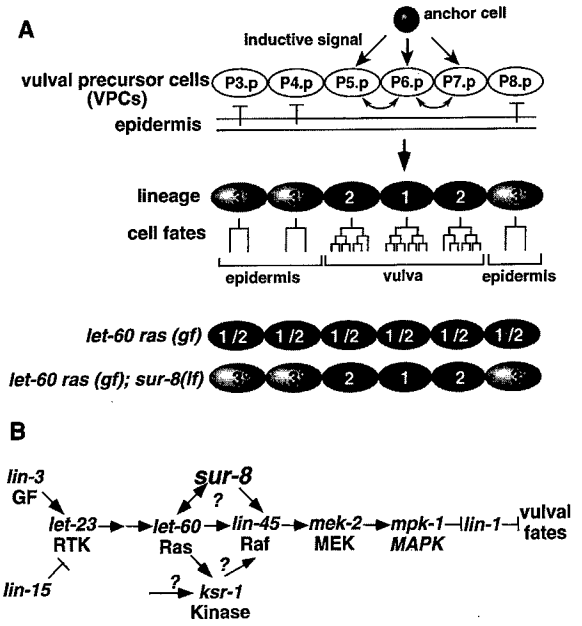


Figure 1. Vulval Cell Fate Specification and the Ras Pathway in *C. elegans*

(A) Wild-type and mutant cell fate specification during vulval development. Vulval precursor cells (VPCs) P3.p through P8.p form an equivalence group, and each cell can take on a vulval cell fate or nonvulval cell (epidermal) fate, depending on the influence of multiple cell signaling events. An inductive signal from the neighboring anchor cell promotes primary vulval fates by activating a Ras pathway; an inhibitory signal from the surrounding hypodermis promotes tertiary fates; and a lateral signal among induced cells promotes secondary fates. In wild type, 3 of 6 VPCs adopt a vulval cell fate, and the pattern of cell fate specification is 3° 3° 2° 1° 2° 3° (100% induction). The activated *let-60 ras* allele, *n1046gf* or G13E, can cause all six VPCs to adopt vulval cell fates (1°/2°), resulting in a Multivulva phenotype and up to 200% induction. Mutations in the *sur-8* gene suppress the Multivulva phenotype caused by activated *let-60 ras* to wild type, resulting in a Suppressed phenotype.

(B) Ras-mediated pathway controlling vulval cell fate specification. Only selected genes involved in the pathway are depicted. Components in this pathway were primarily identified by genetics (Sundaram and Han, 1996; Kornfeld, 1997). *ksr-1* encodes a novel kinase that acts to positively regulate the Ras pathway (Kornfeld et al., 1995; Sundaram and Han, 1995). *sur-8* also functions to positively regulate the Ras pathway and may either define a branchpoint that feeds directly out of *let-60 ras* or be involved with the establishment or maintenance of *let-60 ras* or *lin-45 raf* activation. GF, growth factor; RTK, receptor tyrosine kinase.

### *sur-8* Positively Regulates Ras Pathway Signaling during Vulval Induction

In a *let-60 ras(+)* background, both *sur-8* alleles displayed wild-type vulval induction (Table 2) and appeared to have no additional obvious developmental defects (data not shown). However, the positive role that *sur-8*

Table 1. Phenotype and Gene Dosage Analysis of *sur-8* Mutant Animals

| # | *sur-8* Genotype[a] | *let-60 ras* Genotype | Phenotype | |
|---|---|---|---|---|
| | | | % Muv(n)[b] | % Induction (n)[c] |
| 1 | + | + | 0 (many) | 100 (many) |
| 2 | + | *gf* | 87 (276) | 154 (27) |
| 3 | *ku167/ku167* | *gf* | 4 (333) | 102 (43) |
| 4 | *ku167/+* | *gf* | 77 (57) | 129 (16) |
| 5 | *ku242/ku242* | *gf* | 7 (328) | 103 (33) |
| 6 | *ku242/+* | *gf* | 84 (175) | 153 (15) |
| 7 | *ku167/ku242* | *gf* | 11 (160) | 103 (31) |
| 8 | *ku167/ku167; mDp1* | *gf* | 90 (200) | 154 (32) |
| 9 | +; *mDp1* | *gf* | 100 (247) | 182 (30) |
| 10 | +; *kuEx83* | *gf* | 100 (200) | 196 (18) |
| 11 | + | *gf/+* | ND | 106 (30) |
| 12 | +; *kuEx83* | *gf/+* | ND | 145 (28) |
| 13 | *ku167/mDf4*[d] | *gf* | 14 (270) | 108 (32) |
| 14 | *+/mDf4*[d] | *gf* | 68 (233) | 136 (22) |

[a] The complete genotypes for each strain are: 1, N2 (wild type); 2, *let-60(n1046)*; 3, *sur-8(ku167) let-60(n1046)*; 4, *sur-8(ku167) unc-24 let-60 (n1046)/+ let-60(sy130) dpy-20* (*sy130* encodes the same G13E substitution as *n1046* [Beitel et al., 1990]); 5, *sur-8(ku242) let-60(n1046)*; 6, *sur-8(ku242) unc-24 let-60(n1046)/let-60(sy130) dpy-20*; 7, *sur-8(ku167) let-60(n1046)/sur-8(ku242) unc-24 let-60(n1046)*; 8, *sur-8(ku167) unc-5 let-60(n1046); mDp1*; 9, *unc-5 let-60(n1046); mDp1*; 10, *let-60(n1046); kuEx83. kuEx83* is a transgene carrying *sur-8*(+) genomic DNA; 11, nontransgenic *let-60(sy130) dpy-20/unc-24* siblings of 12; 12, *let-60(sy130)dpy-20/unc-24; kuEx83*; 13, *unc-5 sur-8(ku167) let-60(n1046)/ dpy-13 mDf4 let-60(n1046)*; 14, *unc-5 let-60(n1046)/let-60(n1046) dpy-13 mDf4*.

[b] Percent Multivulva was determined by scoring adult hermaphrodites for presence of ventral protusions under a dissecting microscope. "n" indicates the number of animals scored. ND, not determined.

[c] Average percentage of VPCs adopting a vulval cell fate per animal. In wild type (100% induction), three of six VPCs are induced.

[d] The *dpy-13* marker is semidominant and, when heterozygous, reduces the ability of *sur-8(ku167)* to suppress *let-60(n1046)*. For comparison, *dpy-13 sur-8(ku167) let-60(n1046)/sur-8(ku167) unc-5 let-60(n1046)* animals were 22% (239) Muv and had 117% (26) average induction.

plays in vulval induction became apparent when examining its effects on Ras-mediated signaling in sensitized genetic backgrounds. First, as described above, *sur-8* mutations could strongly suppress both the Muv and male mating defects caused by the *let-60 ras(n1046gf)* allele. Second, an extrachromosomal array containing multiple copies of the cloned *sur-8* gene enhanced the average vulval induction of both *let-60(n1046gf)/let-60(n1046gf)* homozygous animals (from 154% to 196%) and *let-60(n1046gf)/+* heterozygous animals (Table 1).

Finally, *sur-8 (ku167)* severely affected vulval induction when other *ras* pathway components were compromised. *sur-8(ku167)* dramatically enhanced Vulvaless and larval lethal phenotypes caused by a weak loss-of-function mutation in *mpk-1* (Wu and Han, 1994). *mpk-1(ku1)* mutants alone display nearly wild-type vulval induction and only 7% rod-like larval lethality, but *sur-8(ku167)* decreased vulval induction to 0% and increased larval lethality to nearly 100% in the double mutants (Table 2). Because *mpk-1* MAP kinase is a component of the main Ras pathway, this observed genetic interaction suggests that *sur-8* is an important positive regulator of the Ras pathway that functions to increase pathway output. Furthermore, *sur-8(ku167)* also showed strong genetic interactions with a loss-of-function mutation in another regulator of the Ras pathway, *ksr-1* (Sundaram and Han, 1995). *ksr-1(ku68)* mutants alone display wild-type vulval induction (100%) and a weak rod-like lethal phenotype (24%). In *sur-8(ku167);ksr-1(ku68)* double mutants, vulval induction was reduced to 4% and the rod-like larval

Table 2. Genetic Interactions between *sur-8* and *ksr-1* or *mpk-1* MAPK Mutations

| Genotype | % Induction[a] | | | | | | % Average Induction (n) | % Lethal[b] (n) |
|---|---|---|---|---|---|---|---|---|
| | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | | |
| N2 (wild type) | 0 | 0 | 100 | 100 | 100 | 0 | 100% (many) | 0 (138) |
| *sur-8(ku167)* | 0 | 0 | 100 | 100 | 100 | 0 | 100% (28) | 0 (244) |
| *sur-8(ku242)* | 0 | 0 | 100 | 100 | 100 | 0 | 100% (26) | 0 (347)[e] |
| *mpk-1(ku1)*[c] | 0 | 0 | 100 | 94 | 100 | 0 | 98% (17) | 7 (229) |
| *mpk-1(ku1); sur-8(ku167)*[c] | 0 | 0 | 0 | 0 | 0 | 0 | 0% (25) | 100 (80) |
| *ksr-1(ku68)*[d] | 0 | 0 | 100 | 100 | 100 | 0 | 100% (15) | 24 (257) |
| *sur-8(ku167); ksr-1(ku68)*[d] | 0 | 0 | 0 | 11 | 0 | 0 | 4% (19) | 85 (164) |

[a] Individual VPCs adopting 1° or 2° fates were scored as induced.

[b] Percent of animals arresting with an early larval rod-like phenotype, characteristic of loss-of-function mutations in many *ras* pathway genes.

[c] *sur-8* was marked with *unc-24*. Unc self-progeny of *ku1; ku167 unc-24/+* +mothers died as early larval rods. Unc escapers were scored for vulval induction. Double homozygotes were almost completely sterile (average brood = 4), and all progeny died as early larval rods.

[d] *ksr-1* was marked with *lon-2*.

[e] Two animals had abnormal vulval morphology.

Table 3. Epistatic Analysis of *sur-8(ku167)* and Multivulva Mutants

| Genotype[a] | % Muv (n) | % Induction (n) |
|---|---|---|
| *let-60(n1046gf)* | 88 (240) | 154 (27) |
| *sur-8(ku167) let-60(n1046gf)* | 4 (333) | 102 (43) |
| *HSP-raf (gf)*[a] | ND | 116 (31) |
| *sur-8(ku167); HSP-raf (gf)* | ND | 125 (28) |
| *mek-2(ku114); HSP-raf (gf)* | ND | 97 (25) |
| *mpk-1(ku1); HSP-raf (gf)* | ND | 99 (28) |
| *lin-15(n765)* | 98 (214) | 190 (24) |
| *sur-8(ku167); lin-15(n765)* | 73 (302) | 142 (21) |
| *sur-8(ku242); lin-15(n765)* | 58 (258) | 133 (23) |
| *lin-1(ar147)* | 100 (154) | ND |
| *lin-1(ar147) sur-8(ku167)* | 100 (184) | ND |

For *HSP-raf(gf)* experiments, transgenic animals were heat shocked for 80 min at 37°C at early L3. For *lin-15* experiments, animals were grown at 19.2°C. ND, not determined.
[a] Construction of the *HSP-raf(gf)* transgene is described in Experimental Procedures.

lethality was increased to 85% (Table 2). This strong genetic interaction between *sur-8* and *ksr-1* suggests that while the function of neither gene is normally required for Ras signaling, their functions are collectively essential.

### *sur-8* Is Likely to Act Downstream of or in Parallel to *ras* but Upstream of *raf*

To determine at which step in the linear Ras pathway *sur-8* may function, we performed epistasis analysis with mutations that cause Muv phenotypes. Data shown in Table 3 indicate that *sur-8* mutations suppress the Muv phenotype caused by *let-60 ras(n1046 gf)* or *lin-15(n765 lf)* but fail to suppress the Muv phenotype of a *raf(gf)* transgene and *lin-1(ar147 lf)*, suggesting that *sur-8* acts downstream of or in parallel to *let-60 ras* but upstream of *lin-45 raf*.

*lin-45 raf* was shown to act downstream of *let-60 ras* in the vulval induction pathway (Han et al., 1993). Animals carrying an activated *raf(gf)* transgene under the control of a heat-shock promoter displayed an Muv phenotype upon heat shock (Table 3). As expected, the Muv phenotype was completely suppressed by weak mutations in either *mek-2* or *mpk-1* (Table 3), which act downstream of *lin-45 raf* (Sundaram and Han, 1996; Kornfeld, 1997). However, a *sur-8* mutation failed to suppress the Muv phenotype caused by the *raf(gf)* transgene. Heat-shocked *raf(gf)* mutants or *sur-8(ku167);raf(gf)* double mutants displayed similar average vulval induction of 116% and 125%, respectively (Table 3), indicating that *sur-8* does not function downstream of *lin-45 raf* in the same linear pathway as *mek-2* and *mpk-1*.

*lin-15* functions upstream of *ras* at the level of *let-23* RTK to inhibit *let-23* signaling (Ferguson et al., 1987). *sur-8* mutations could suppress the *lin-15* mutant Muv phenotype (Table 3). However, this suppression was not complete, possibly due to the inability of *sur-8* mutations to overcome strong pathway activity caused by the *lin-15(n765)* mutation. *lin-1* is a negative regulator acting downstream of *mpk-1* MAP kinase (Wu and Han 1994; Beitel et al., 1995). A loss-of-function mutation of *lin-1, ar147* causes a 100% Muv phenotype that is not suppressed by *sur-8(ku167)* at all (Table 3). The interaction between the *sur-8* mutations and the *lin-15* or *lin-1* mutation is consistent with the suggestion that *sur-8* acts downstream of or in parallel to Ras, but upstream of Raf.

### *sur-8* Function Is Required during Vulval Induction

Vulval cell fate specification takes place at the end of the L2 stage, after the anchor cell is born and before the VPCs undergo their first division (Kimble, 1981). To determine if *sur-8(+)* activity is required at this stage for proper vulval induction, we assayed the ability of *sur-8(+)* to rescue the suppression phenotype of *sur-8(ku167) let-60(n1046gf)* animals at various stages of development. We generated transgenic *sur-8(ku167) let-60(n1046gf)* animals carrying a *sur-8* cDNA (see below) under the control of a heat-inducible promoter and subjected them to heat shock at different developmental stages. Control transgenic animals without heat shock displayed a slightly rescued phenotype of 123% vulval induction (data not shown), probably resulting from leaky *sur-8* expression from the heat shock promoter. Animals heat shocked before or during vulval induction (between early L2 and mid L3 stages) displayed a fully rescued phenotype, resulting in over 160% induction (Table 4), similar to that observed in *sur-8(ku167) let-60(n1046gf)* mutants carrying a transgene of *sur-8* under

Table 4. Functional Tests of Human or *C. elegans sur-8* cDNA under Control of a Heat Shock Inducible Promoter

| Transgene[a] | Stage | Induction % | | | | | | Total Induction | n |
|---|---|---|---|---|---|---|---|---|---|
| | | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | | |
| vector | eL3 | 0 | 8 | 100 | 100 | 100 | 6 | 103% | 38 |
| *Ce sur-8* | eL3 | 61 | 97 | 100 | 100 | 100 | 93 | 184% | 42 |
| *Hs sur-8* | eL3 | 16 | 40 | 100 | 100 | 100 | 70 | 142% | 25 |
| *Ce sur-8* | L1 | 10 | 55 | 100 | 100 | 100 | 26 | 133% | 19 |
| *Ce sur-8* | eL2 | 28 | 81 | 100 | 100 | 100 | 76 | 162% | 21 |
| *Ce sur-8* | mL3 | 33 | 83 | 100 | 100 | 100 | 89 | 169% | 19 |
| *Ce sur-8* | eL4 | 3 | 24 | 100 | 100 | 100 | 28 | 134% | 29 |

*sur-8(ku167) let-60(n1046)* hermaphrodites carrying the indicated transgene were heat shocked at the indicated stage (e = early, m = mid) at 37°C and scored for vulval induction at stage L4 or as young adults. Heat shock was for 80 min for the upper set of experiments and 40 min for the lower set of experiments.
[a] Extrachromosomal arrays carried either full-length Ce *sur-8* coding region or Hs *sur-8* coding region under the control of a heat shock inducible HSP-16 promoter.

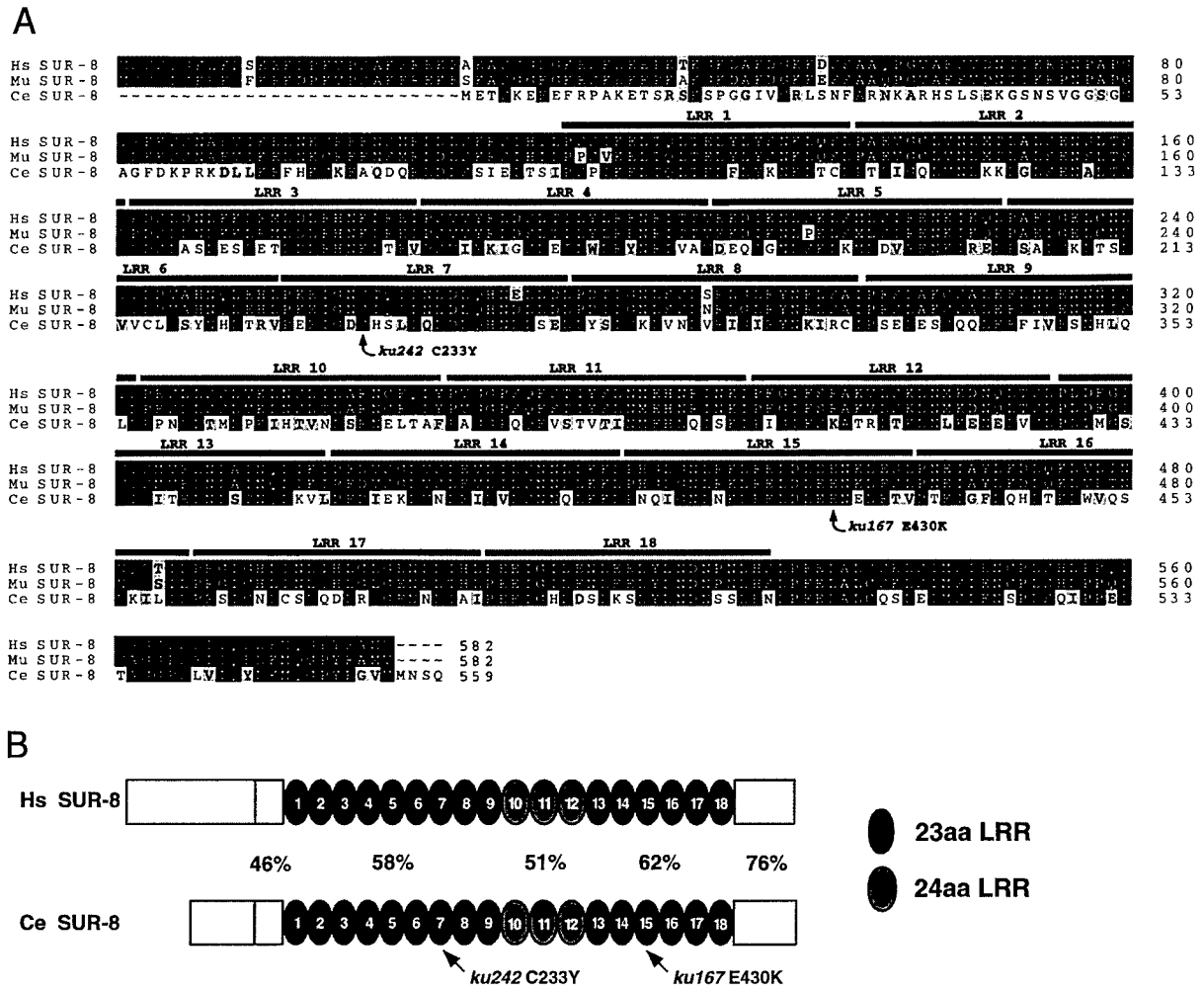


Figure 2. Comparison of Sequence and Protein Structure of Mammalian and *C. elegans SUR-8*

(A) Complete amino acid alignment of predicted SUR-8 protein sequences from human (Hs SUR-8), mouse (Mu SUR-8), and *C. elegans* (Ce SUR-8). Residue identity between species is highlighted in black, and similarity is highlighted in gray. The positions of the leucine-rich repeats (LRRs) are indicated with dark bars (for 23 amino acid repeats) or light bars (for 24 amino acid repeats). The positions of the amino acid substitutions are indicated for the two *sur-8* mutations.

(B) Comparison of *C. elegans* and human SUR-8 protein structure. Positions of the leucine-rich repeats and the amino acid substitutions of *sur-8* mutants are shown. Percent amino acid identity between *C. elegans* and human SUR-8 is indicated for the domains shown. The N-terminal 93 amino acids of human SUR-8 shares no sequence homology with *C. elegans* SUR-8. Human and mouse SUR-8 share 98% amino acid identity.

control of its own promoter (Table 1). In contrast, animals heat shocked either in L1, before the anchor cell is born, or in L4, after Pn.p cells have executed their fate, displayed only a partially rescued phenotype of 133% or 134% vulval induction (Table 4). The rescuing activity observed in early L2 heat-shocked animals is most likely due to SUR-8 protein perdurance. Thus, *sur-8(+)* activity is required before or during the time of vulval cell fate specification for vulval development but is not required at earlier or later times.

### *sur-8* Encodes a Novel Leucine-Rich Repeat Protein

We cloned *sur-8* by genetic mapping followed by transformation-rescue (see Experimental Procedures). *sur-8* was mapped to position 1.86 on chromosome IV between the markers *dpy-13* and *unc-5*. Cosmids containing genomic DNA from this region were tested for *sur-8(+)* activity by assaying their ability to revert the Suppressed phenotype of *sur-8(ku167) let-60(n1046gf)* animals back to Muv. A single cosmid, AC7, contained complete rescuing activity, as did a 12kb AC7-derived subclone (data not shown). The subclone was predicted to contain a single gene, designated AC7.1 by the *C. elegans* genome sequencing project. A full-length 2.1 kb cDNA was identified by screening a mixed stage library (gift from Peter Okkema), using a genomic probe derived from the predicted AC7.1 gene. Northern blot analysis indicated that this cDNA was the only transcript encoded by *sur-8* (data not shown). We conclude that the gene defined by the isolated cDNA corresponds to

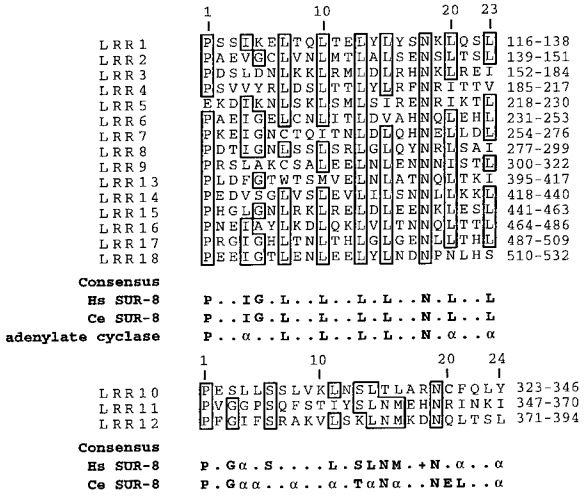


Figure 3. Sequence Alignment of SUR-8 Leucine-Rich Repeats

Alignment of 23 amino acid and 24 amino acid LRRs of Hs SUR-8 is shown. Consensus amino acids are boxed and shown below with the consensus of Ce SUR-8 LRR and yeast adenylate cyclase (yeast A.C.) LRR. α, aliphatic residue (A, V, L, I, F, Y, or M).

*sur-8* because missense mutations from *sur-8* mutant DNA are located in the coding region of the cDNA and because this cDNA was able to rescue *sur-8* mutants (see below).

The 1.7 kb coding sequence of *sur-8* is predicted to encode a novel 559 amino acid protein, containing 18 tandem repeats of the leucine-rich repeat (LRR) motif (amino acids 89–505) (Figure 2). LRRs, characterized by a consensus composed of leucines at invariant positions, are found in a variety of proteins with diverse biological functions and are proposed to mediate protein–protein interactions (Kobe and Deisenhofer, 1994). Fifteen of the 18 SUR-8 LRRs are 23 amino acids long and form a consensus that is similar to that of yeast adenylate cyclase LRRs (Figure 3). These 23 amino acid LRRs form two tandem clusters of nine and six repeats that are separated by three tandem LRRs that are 24 amino acids long and form a distinct consensus with no obvious similarity to other known LRR motifs. SUR-8 contains N-terminal and C-terminal non-LRR flanking sequences of 88 and 53 amino acids, respectively (Figure 2).

We have identified a missense mutation associated with each *sur-8* allele (Figure 2). *sur-8(ku242)* encodes a cysteine 233 to tyrosine substitution in a consensus position within LRR 7. *sur-8(ku167)* encodes a glutamic acid 430 to lysine substitution in a nonconsensus position within LRR 15. Both mutations were found to alter amino acids conserved in mammalian *sur-8* homologs (Figure 2, and see below), indicating that these residues may have an evolutionarily conserved function.

### *C. elegans sur-8* Is Structurally and Functionally Conserved in Mammals

An expressed sequence tag (EST) database search revealed several overlapping human and mouse ESTs that shared from 49% to 70% amino acid identity with the non-LRR C-terminal sequences of Ce SUR-8. We used primers derived from a human or mouse EST (GenBank accession numbers W51818 and AA286839, respectively) to amplify the 5′ ends of the cDNAs by performing 5′ RACE from human brain cDNA or mouse liver cDNA (Clonetech). Sequences from the 5′ RACE and EST clones were compiled to generate the full-length (4.1 kb) human and mouse *sur-8* cDNAs. Multitissue Northern blot (Clonetech) analysis using a probe derived from the human cDNA revealed that this cDNA corresponded to a single transcript of the predicted size, and the transcript was detected in all tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (data not shown). The predicted proteins encoded by the human and mouse cDNAs are 98% identical at the amino acid level.

Comparison of the amino acid sequences encoded by the mammalian and *C. elegans sur-8* genes revealed significant homology in amino acid sequence and overall protein structure. Mammalian SUR-8 contains exactly the same number of LRRs with the identical length and organization as *C. elegans* SUR-8 LRRs (Figures 2 and 3). The LRR regions share 58% identity and the C-terminal extensions share 76% identity, while the N-terminal extensions share very little identity (Figure 2). The 23 amino acid repeats in Hs SUR-8 have the same consensus sequence as their Ce SUR-8 counterparts (Figure 3).

We found that in addition to sharing a high degree of structural homology, the *C. elegans* and human SUR-8 proteins share functional homology. Human *sur-8* cDNA expressed under the control of a heat shock inducible promoter was able to rescue the mutant phenotype of *sur-8(ku167) let-60(n1046gf)* animals (Table 4). Hs *sur-8* could revert the Suppressed phenotype from 103% induction to 142% induction. Control animals expressing Ce *sur-8* cDNA displayed a fully rescued phenotype with 184% induction (Table 4). Because Hs *sur-8* could provide *sur-8(+)* activity in *sur-8* mutants, we conclude that Hs *sur-8* is a functional homolog of Ce *sur-8*.

### SUR-8 Interacts with LET-60 RAS, but Not with a LET-60 RAS Effector Domain Mutant

Yeast adenylate cyclase contains 26 LRRs (Kataoka et al., 1985) that are required for binding to and activation by Ras during vegetative yeast growth (Field et al., 1990; Suzuki et al., 1990). The observation that SUR-8 LRRs form a consensus that is similar to that of yeast adenylate cyclase LRRs led us to test the interaction between SUR-8 and several Ras pathway components. Using the yeast two-hybrid system, while we failed to detect an interaction between SUR-8 and wild-type LIN-45 Raf, MEK-2 MEK, MPK-1 MAP kinase, or KSR-1 (data not shown), we detected an interaction with wild-type LET-60 Ras, as assayed by the activation of a His reporter (Figures 4 and 5A) and a lacZ reporter (data not shown).

Given that mutations in the LRR regions result in loss of *sur-8* function, we examined the effect of these mutations on LET-60 RAS interaction. Interestingly, while *ku167* E430K had no effect on LET-60 Ras interaction, *ku242* C233Y eliminated detectable interaction with LET-60 Ras (Figure 4A), even though both mutant proteins were expressed at similar levels in yeast (data not shown). The *ku242* C233Y mutation is in LRR 7 of the

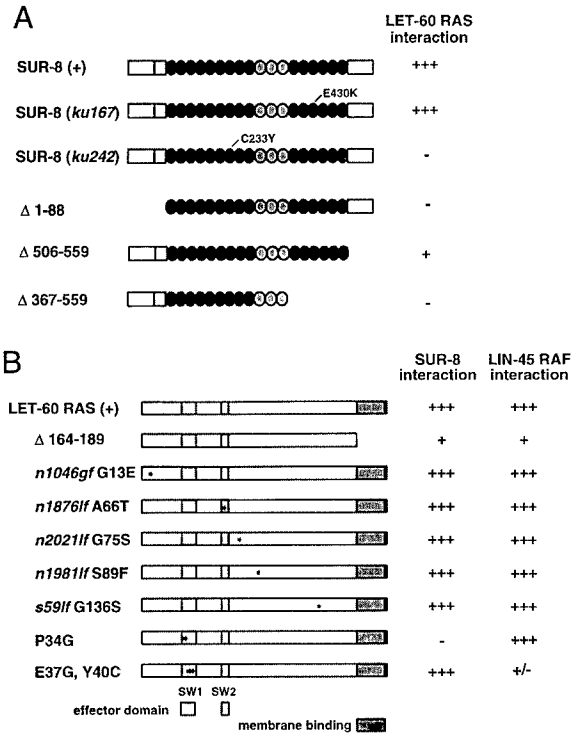


Figure 4. Yeast Two-Hybrid Interactions between SUR-8 and LET-60 RAS

LET-60 Ras proteins fused to the GAL4 DNA binding domain were expressed together with SUR-8 proteins fused to the GAL4 activation domain in a yeast reporter strain and assayed for interaction by growth on $His^-$ selective media.

(A) Summary of interaction between LET-60 RAS and Ce SUR-8 mutants. Different SUR-8 domains and sites of point mutations encoded by the sur-8 mutants are shown, as in Figure 2. Each construct shown was expressed as a fusion protein at similar levels in yeast as shown by Western blot analysis (data not shown). Abbreviations: +++, colony growth on $His^-$ medium within 3 days; +, colony growth within 5 days; +/−, very slow colony growth after 5 days; −, no colony growth after 5 days.

(B) Summary of interaction between Ce SUR-8 or LIN-45 RAF and LET-60 RAS mutants. Ras functional domains are shaded. Previously identified LET-60 Ras point mutations are depicted with an allele number (Beitel et al., 1990) and positions shown with an asterisk. Abbreviations same as in (A); SW1 is switch 1 and SW2 is switch 2.

N-terminal LRR cluster, and these data demonstrate that this residue is critical for both SUR-8 function and LET-60 Ras binding.

To define a specific region of SUR-8 involved in LET-60 Ras binding, we tested SUR-8 deletion mutants for LET-60 Ras interaction (Figure 4A). Deletion of the N-terminal 88 amino acid nonLRR region resulted in elimination of LET-60 Ras binding. Similarly, deletion of the C-terminal 53 amino acid non-LRR sequence resulted in reduction of LET-60 Ras binding. In addition, deletion of the last six LRRs, including repeat 15, abolished LET-60 Ras binding. It is likely that N- or C-terminal deletions alter global protein structure that may result in decreased LET-60 Ras interaction.

We next wanted to define a domain of LET-60 Ras required for SUR-8 interaction. We tested interaction of SUR-8(+), and as a control, LIN-45 Raf, with several different LET-60 Ras mutants using the yeast two-hybrid system. Mutations tested included point mutations that cause phenotypes in *C. elegans* (Beitel et al., 1990), deletion mutations, and effector domain mutations (Figure 4B). Point mutations in the effector domain of H-Ras have been shown to abolish binding to several putative Ras binding proteins, including Raf1, PI-3 kinase, and Ral-GDS (Rodriguez-Viciana et al., 1997).

All of the LET-60 Ras loss-of-function mutations tested had no effect on SUR-8 or LIN-45 Raf binding. In addition, the mutation encoded by the gain-of-function allele, *n1046gf* G13E, had no effect on SUR-8 binding or LIN-45 Raf binding. Deletion of the membrane targeting region had only a slight effect on SUR-8 binding or LIN-45 Raf binding. An effector domain double mutation, E37G Y40C, interfered with LIN-45 Raf binding but had no effect on SUR-8 binding (Figure 4B), suggesting that SUR-8 does not share binding specificity with Raf.

More interestingly, we identified one effector domain mutation, P34G, that specifically interfered with SUR-8(+) binding but had no effect on LIN-45 Raf binding. A P34G Ras mutant has previously been shown to bind Raf1 with wild-type affinity in vitro but to fail to cause transformation or induce neurite outgrowth in vivo (Akasaka et al., 1996). SUR-8 is thus a likely candidate for promoting full Ras activity through binding the Ras effector domain at a site that is distinct from that of Raf.

### Hs SUR-8 Interacts with N-Ras and K-Ras 4B but Not H-Ras In Vitro

Given the functional and structural homology between Ce *sur-8* and Hs *sur-8*, we were interested in determining whether Hs SUR-8 could bind mammalian Ras. We tested the interaction of Hs SUR-8 with three human Ras family members, N-Ras, K-Ras 4B, and H-Ras, in the yeast two-hybrid system. We detected a strong interaction between Hs SUR-8 and two family members, K-Ras and N-Ras, but only a weak interaction with H-Ras (Figure 5A). As a control for Ras expression, we showed that all three Ras family members interacted strongly with Raf1 (Figure 5A). In addition, SUR-8 and Ras displayed cross-species interactions, reinforcing the idea that *sur-8* function may be evolutionarily conserved.

These observations were confirmed by testing in vitro interaction of Hs SUR-8 and Ras family members (Figure 5B). Bacterially expressed GST-Ras fusion proteins were purified and tested for their ability to interact with purified Hs SUR-8 or Raf1. While all three family members bound Raf1 with similar affinities, only N-Ras and K-Ras were capable of binding Hs SUR-8 strongly. However, while Raf1 bound Ras with GTP dependence, Hs SUR-8 showed no GTP dependence for Ras binding. Both GDP- and GTP-loaded Ras proteins bound Hs SUR-8 with similar affinities. Thus, SUR-8 displayed a differential binding specificity for individual Ras family members that appeared not to depend on the activation state of Ras.

## Discussion

In this study, we describe the identification of *sur-8*, a novel regulator of the Ras-mediated signal transduction

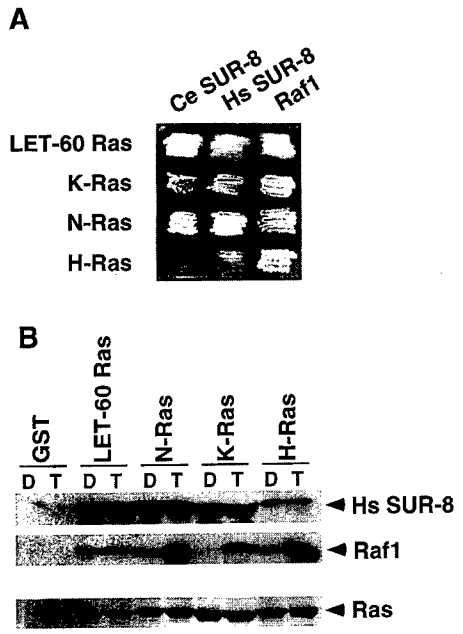


Figure 5. Interaction between SUR-8 and Ras Family Members

(A) Yeast two-hybrid system interaction of Ce SUR-8, Hs SUR-8, or human Raf1 with LET-60 Ras or human Ras family members. The ability of yeast to grow on $His^-$ plates indicated interaction of the fusion proteins indicated. Interaction with Raf1 was tested as a control for Ras expression.

(B) In vitro binding of Hs SUR-8 with human Ras family members. GST-Ras fusion proteins were loaded with GDP (D) or GTP (T) and were incubated with either full-length Hs SUR-8 or Raf1 (residues 1–269). Bound Hs SUR-8 or human Raf1 was subjected to SDS-PAGE and Western immunoblotting with an anti-5× His monoclonal antibody. As a control for amount of Ras in the binding assays, one-eighth of the amount of Ras input in the binding reactions is shown in the lowest panel.

pathway during vulval induction, and we provide genetic evidence that *sur-8* acts to positively regulate Ras pathway signaling. In addition, we show that *sur-8* encodes a highly conserved, novel, LRR-containing protein that binds to a subset of Ras family members in vitro. These findings suggest that *sur-8* defines a regulatory branch-point in the Ras pathway, and *sur-8* may either be involved in regulating *raf* activity or be a *ras* target.

### *sur-8* Positively Regulates *let-60 ras* Signaling

Several lines of genetic data indicate that the normal function of *sur-8* is to positively regulate Ras pathway signaling during cell fate specification. First, loss-of-function mutations in *sur-8* can suppress the phenotypes of an activated *let-60 ras* mutation. Second, addition of *sur-8(+)* gene copies from either a duplication or injected transgenes enhances the Multivulva phenotype caused by the activated *ras* mutation. Third, mutations in *sur-8* dramatically enhance the Vulvaless and larval lethal phenotypes caused by a partial loss-of-function mutation of *mpk-1*, indicating that *sur-8* is required for the maximal strength of signaling activity. Finally, a *sur-8* mutation synergizes with a loss-of-function mutation in *ksr-1*, indicating that *sur-8* and *ksr-1* functions are collectively essential for Ras-mediated signal transduction even when the main pathway is wild type.

*sur-8* is likely to regulate *ras*-mediated cell specification events in multiple tissues. Mutations in *sur-8* suppress the male mating defect caused by activated *ras* mutations, dramatically enhance the rod-like larval lethal phenotypes caused by mutations in *ksr-1*, and enhance lethality and sterility caused by *mpk-1* mutations. The male tail defects of *let-60 ras* were shown to be the result of misspecification of B blast cell fates (Chamberlin and Sternberg, 1994; Yochem et al., 1997). The rod-like larval lethal phenotype of loss-of-function mutations in *let-60 ras*, and likely its downstream target genes, are caused by misspecification of the excretory duct cell (Yochem et al., 1997). The sterile phenotype of *let-60 ras* and *mpk-1* mutations is a result of defects in germ cell nuclei exiting from pachytene (Church et al., 1995).

Genetic epistasis experiments demonstrate that mutations in *sur-8* can suppress an activated *ras* allele but not an activated *raf* transgene. This analysis indicates that *sur-8* functions genetically downstream of or in parallel to *let-60 ras* but not downstream of *lin-45 raf*, which is consistent with our molecular analysis indicating that SUR-8 directly interacts with Ras. Since Raf is a direct target of Ras (Moodie et al., 1993), SUR-8 may function either as part of a complex to affect Ras-Raf activation or in a branch feeding into or out of the pathway at the level of Ras and Raf. *ksr-1* has the same epistatic relationship as *sur-8* with the Ras pathway (M. Sundaram and M. H., unpublished), but because mutations in *ksr-1* synergize with mutations in *sur-8*, we believe that these genes are not acting on each other to stimulate signaling, but rather are acting at distinct points.

Because loss-of-function *sur-8* mutations have no effect on vulval induction in a wild-type background, we propose that the normal function of *sur-8* is to increase signaling output of the inductive signal-activated Ras pathway. Alternatively, *sur-8* function may be essential in Ras-mediated signaling, but its function is redundant with another gene that performs the same role in Ras-mediated signaling.

### SUR-8 Is a Structurally and Functionally Highly Conserved, Novel LRR Protein

*sur-8* is predicted to encode a novel protein composed largely of leucine-rich repeats. SUR-8 contains 18 tandem LRRs. The 23 amino acid repeats from two clusters of 9 and 6 repeats separated by the three 24 amino acid repeats. We have cloned a human and a mouse SUR-8 homolog, which themselves are greater than 98% identical and share high sequence homology and overall protein organization with Ce SUR-8. In addition to sharing a highly conserved protein structure, we have shown that Hs *sur-8* can complement a Ce *sur-8* mutation (Table 4), indicating that *sur-8* function in Ras signaling is evolutionarily conserved.

Leucine-rich repeats are protein motifs of 20–28 amino acids, characterized by a core consensus consisting of invariantly spaced leucines and asparagine (LxxLxLxxN). LRRs have been found in many functionally diverse proteins in a variety of organisms and have been shown to mediate protein–protein interactions (Kobe and Deisenhofer, 1994). The crystal structure of porcine ribonuclease inhibitor, which like SUR-8 is almost completely

composed of LRR (Hofsteenge et al., 1988), has been determined (Kobe and Deisenhofer, 1993). It forms a nonglobular, horseshoe-like structure with the α-helical portion of each repeat aligned in parallel and exposed to the outer surface and the β-strand portion of each repeat exposed to the inner circumference (Kobe and Deisenhofer, 1994). The mutation encoded by *sur-8(ku167)* is a charge reversal in a negatively charged region predicted to form a β sheet, while the mutation encoded by *sur-8(ku242)* lies in a predicted α-helical region.

Yeast adenylate cyclase functions to regulate vegetative growth in *S. cerevisiae* and contains 26 LRRs. In *S. cerevisiae*, but not in higher eukaryotes or in *S. pombe*, adenylate cyclase interacts directly with Ras, and this binding is required for adenylate cyclase activation and cAMP production. Ras binding is GTP dependent and is disrupted by effector domain mutations, indicating that adenylate cyclase is a Ras effector in *S. cerevisiae*. This binding is mediated at least in part by LRRs since mutations in LRRs disrupt Ras binding (Field et al., 1990; Suzuki et al., 1990). Notably, LRRs are not found in eukaryotic adenylate cyclase, and the consensus formed by the LRR of yeast adenylate cyclase is similar to that formed by the 23 amino acid LRRs of SUR-8 (Figure 3).

### SUR-8 May Function through Binding to the Ras Effector Domain

Our findings indicate that both Ce SUR-8 and Hs SUR-8 interact with Ras in the yeast two-hybrid system and in vitro (Figure 5). This interaction is specific to Ras, since no interactions were detected between Ce SUR-8 and other Ras pathway components tested (data not shown). Mutation of cysteine 233 in LRR7 residue to tyrosine in the *ku242* loss-of-function mutant completely blocks LET-60 Ras interaction (Figure 4A). The correlation between loss of *sur-8* function and loss of LET-60 Ras binding suggests that SUR-8 binding to LET-60 Ras is necessary for optimal *sur-8* function. In contrast, a mutation in glutamic acid 430 in LRR 15 encoded by *ku167* has no effect on LET-60 Ras binding. Because the substitutions encoded by *ku242* and *ku167* are located in separate LRR clusters, it is tempting to speculate that SUR-8 acts as an adaptor protein, binding Ras through one LRR cluster and binding another unidentified protein through the other.

Mutational analysis of LET-60 Ras suggests that SUR-8 and Raf have different effector domain binding specificities. A P34G mutation blocks interaction of LET-60 Ras with SUR-8 but not with LIN-45 Raf. In contrast, mutation of residues E37 and Y40 interferes with interaction between LET-60 Ras and LIN-45 Raf but not between LET-60 Ras and SUR-8 (Figure 4B). Mutations of residues 37 or 40 in H-Ras disrupt Raf1 binding and Ras function (White et al., 1995). The P34G mutation abolishes the ability of Ras to transform NIH3T3 cells and to induce neurite outgrowth while maintaining Raf binding ability (Akasaka et al., 1996). These observations suggest a second requirement for Ras activation in addition to Raf binding, which is possibly dependent on interaction with SUR-8.

The P34G mutation has also been shown to disrupt the interaction of yeast adenylate cyclase with Ras (Akasaka et al., 1996). Yeast adenylate cyclase contains 28 tandem LRRs, which are required for binding to and activation by Ras. Thus, LRR-mediated interaction with Ras may be an evolutionarily conserved mechanism for Ras-effector interactions. SUR-8 LRRs are similar to those found on Rsp-1, which was identified as a multicopy suppressor of K-*ras* transformed cells (Cutler et al., 1992). Our preliminary data indicate that Rsp-1 competes with SUR-8 for Ras binding (unpublished observations), raising the intriguing possibility that Rsp-1 overexpression inhibits Ras transformation by blocking a functionally significant SUR-8–Ras interaction.

### Hs SUR-8 Binds a Subset of Ras Family Members

Hs SUR-8 specifically binds to K-Ras 4B and N-Ras, but binds only weakly to H-Ras, in vitro and in the yeast two-hybrid system (Figures 5A and 5B). The in vitro studies suggest that binding is direct. Given the probability of unique roles for different Ras family members, it is possible that SUR-8 is involved in generating a functional specificity for some Ras family members. Intriguingly, Ras binding to SUR-8 does not appear to be GTP dependent. Hs SUR-8 binds both GTP- and GDP-bound N-Ras and K-Ras in vitro. In contrast, Raf1 binds all three Ras family members with GTP dependence. One possible model for SUR-8 function is that it is involved in the establishment or maintenance of Ras activity by facilitating binding or activation of Ras effectors, such as Raf. Indeed, the mechanisms by which Raf is activated upon membrane recruitment are poorly understood. Alternatively, SUR-8 may mediate its positive effects on Ras activation by inhibiting the activity of a negative regulator of Ras, such as GTPase activating protein, GAP. Finally, SUR-8 may be an adaptor protein for effectors distinct from Raf that act in a branched pathway. Further molecular and biochemical studies should elucidate the evolutionarily conserved role of SUR-8 function in the Ras-mediated signaling process.

## Experimental Procedures

### *C. elegans* Strains and Phenotypic Analysis

N2 and derivative strains were maintained as described by Brenner (1974) and grown at 20°C unless otherwise indicated. Unless otherwise indicated, the reference for alleles is Riddle et al. (1997). LGI: *mek-2(ku114)*. LGIII: *mpk-1(ku1)*, *dpy-17(e164)*, and *unc-119(ed3)*. LGIV: *sur-8(ku167)*, *sur-8(ku242)*, *unc-24(e138)* *unc-5(e53)*, *dpy-13(e184)*, *unc-17(e113)*, *lin-1(ar147)*, *lin-45(ku112)* (D. Green and M. H., unpublished), *dpy-20(e1282)*, *let-60(n1046)*, *let-60(sy130)*, *mDf4*, and *mDp1(IV;f)* (Rogalski and Riddle, 1988). LGV: *him-5(e1490)*. LGX: *lon-2(e678)*, *ksr-1(ku68)*, *lin-15(n765)*, and *xol-1(y9)*.

Multivulva (Muv) and Egg-laying defective (Egl) phenotypes were scored as described previously (Ferguson and Horvitz, 1985). Percent larval lethality (Let) was determined by collecting eggs from gravid hermaphrodites for 1–2 hr and examining plates for arrested rod-like larvae. Vulval induction was determined by examining the number and locations of VPC descendant nuclei of early L4 larvae under Nomarski optics as described previously (Han et al., 1990). Average vulval induction was scored as 100% if 3 of 6 VPCs were induced (wild type), 0% if 0 of 6 were induced (Vulvaless), or 200% if 6 of 6 VPCs were induced (Muv).

### Transgenic *let-60 ras(n1046gf)* Suppressor Screen and Isolation of *sur-8* Mutants

The transgenic strain used to screen for suppressors of *let-60(n1046)* carried the integrated array *kuIs14*, which contains the

*let-60(n1046)* genomic DNA (pMH132) and *dpy-20(+)* genomic DNA (pMH86) (Sundaram et al., 1996). Transgenic L4 hermaphrodites were mutagenized with 50 mM ethylmethane sulfonate (EMS) (Brenner, 1974) and F1 and F2 self-progeny were screened for non-Muv animals. Non-Muv animals that produced nearly all non-Muv progeny (less than 5%) were outcrossed once to *let-60(sy130) dpy-20(1282)*, and Dpy non-Muv progeny were outcrossed several times to *let-60(n1046)*. The *let-60(n1046); sup* strains were mapped using 2- and 3-factor mapping methods (Brenner, 1974). Complementation tests were performed with *mek-2* and *lin-45* alleles.

*sur-8(ku167)* isolated from this screen was outcrossed seven times and 3-factor mapped using *dpy-13* and *unc-5* on LGIV. Of Dpy non-Unc recombinants, 46 of 52 had the genotype *dpy-13 sur-8(ku167) let-60(n1046)*, and 2 of 18 Unc non-Dpy recombinants had the genotype *sur-8(ku167) unc-5 let-60(n1046)*, placing *sur-8* at map position *1.72* of LG IV.

*sur-8(ku242)* was isolated from a noncomplementation screen. *unc-24 let-60(n1046); lon-2(e678) xol-1(y9)* or *unc-17(e113) let-60(n1046); lon-2 xol*-1 L4 hermaphrodites were EMS mutagenized and mated to *sur-8(ku167) let-60(n1046); him-5* males. We screened approximately 10,000 haploid genomes. Non-Muv F1 cross progeny were isolated, and those that continued to segregate less than 10% Muv progeny were outcrossed to a *let-60(n1046)* strain. *sur-8(ku242)* was unlinked from the Unc mutations by outcrossing with *let-60(n1046)* and picking suppressed non-Unc recombinants. *sur-8(ku242)* was outcrossed with *let-60(n1046)* an additional three times.

Because *mDf4* caused no lethality when in *trans* to *sur-8(ku167)*, the noncomplementation screen should not have been biased against isolating null alleles. One probable explanation for the lower frequency at which *sur-8* loss-of-function alleles were isolated is that we restricted the characterization of suppressors to those that suppressed to below 5% Muv, which might be too low to isolate many other *sur-8* mutations.

### Dosage Analysis

*mDf4* is linked to the semidominant *dpy-13* allele, *e184* (Rogalski and Riddle, 1988). For deficiency analysis, *mDf4* was linked to *let-60(n1046)* by selecting Muv semi-Dpy recombinants from *dpy-13 mDf4/unc-5 let-60(n1046)* heterozygotes. The recombinant *dpy-13 mDf4 let-60(n1046)* chromosome was balanced with nT1 and used to do a complementation test with either *unc-5 sur-8(ku167) let-60(n1046)* or *unc-5 let-60(n1046)* by scoring non-Unc, semi-Dpy cross progeny [genotype: *unc-5 +/- sur-8(ku167) let-60(n1046); dpy-13 mDf4 let-60(n1046)*] for percent Muv and percent induction.

*mDp1* is a free duplication that covers *unc-17, dpy-13, sur-8*, and *unc-5* but not *let-60.* For the duplication analysis, *dpy-13 unc-5 let-60(n1046);mDp1* was constructed and tested for rescue of the Suppressed phenotype by crossing with either *sur-8(ku167) unc-5 let-60(n1046)* or *unc-5 let-60(n1046)*. Progeny segregating no Dpy [genotype: *sur-8(ku167) unc-5 let-60 (n1046); mDp1*] were scored for percent Muv and percent vulval induction.

### Construction of Double Mutants and Transgenic Strains

Double mutants were constructed using standard genetic methods, and markers used are indicated in the tables. For the *sur-8(ku167); lin-15, sur-8(ku242); lin-15, sur-8(ku167);raf(gf)* double mutants and for *sur-8(ku167)* and *sur-8(ku242)* single mutants, the presence of *sur-8* mutations was confirmed by sequencing the appropriate region of *sur-8* genomic DNA from each strain.

Because *mpk-1(ku1); unc-24 sur-8(ku167)* double mutants were larval lethal, double homozygotes were derived from mothers that were heterozygous for *sur-8(ku167)*. Occasionally, Unc segregants were observed that were examined under Nomarski optics for vulval induction and replated to observe progeny. Unc animals either represented escapers, which were 0% induced and had no viable progeny, or represented recombinants, which were 100% induced and segregated viable progeny.

Transgenic strains were generated by germline transformation as described previously (Mello et al., 1991). Germline rescue: cosmids spanning the *sur-8* region were obtained from A. Coulson (Sanger Center). 5 μg/ml of single cosmids or subclones were coinjected with 40 μg/ml of the *unc-119* transformation marker pDP#MM016 (Maduro and Pilgrim, 1995) into *unc-119; sur-8(ku167) let-60(n1046)* animals, and non-Unc stable lines were analyzed. The cosmid AC7 rescued the Suppressed phenotype to between 50% and 100% Muv in 4 of 6 stable lines generated. pDS12 contained a 13 kb PstI-SacII AC7 subfragment cloned into pBluescript (Stratagene) and rescued to 100% Muv in 3 of 3 stable lines generated. *kuEx83* is a transgene containing pDS12 (injected at 5 μg/ml) and a *sur-5* promoter::*gfp* reporter construct, pTG96.1 (injected at 100 μg/ml).

raf(gf) ***Epistasis***

*kuIs17* is a transgene containing *raf(gf)* (pMS88) and *dpy-20(+)* genomic DNA (pMH86, [Han and Sternberg, 1990]) integrated into the genome. pMS88 contains a Drosophila *raf* gain-of-function mutant gene cloned into the HSP16–41 vector pPD49.83 (gift from A. Fire). In this *raf(gf)* gene, the kinase domain of Draf is fused to the transmembrane domain of the Torso receptor (Dickson et al., 1992).

***Heat Shock Rescue***

pDS23 and pDS25 contain Ce *sur-8* cDNA and Hs *sur-8* cDNA, respectively, cloned into the NheI and KpnI sites of pPD49.83. Either pDS23 (10 μg/ml), pDS25 (20 μg/ml), or pPD49.83 (10 μg/ml) was coinjected with pUnc-119 (40 μg/ml) into *unc-119 sur-8(ku167) let-60(n1046)*, and non-Unc stable lines were analyzed. Three pDS23 and two pDS25 bearing independent lines displayed similar vulval phenotypes upon heat shock.

### sur-8 cDNA Cloning and Allele Sequencing

A 2kb HindIII genomic subclone containing part of the AC7.1 sequence (pDS7) was used as a probe for a mixed stage Northern, identifying a single band of 2.2 kb. This fragment was used to probe a λgt11 mixed stage *C. elegans* cDNA library (gift from P. Okkema). From approximately 1 million plaques screened, 10 positive clones were isolated and their inserts were PCR amplified. The two largest inserts were sequenced using an ABI automated sequencer and were found to each have a 5′ UTR, a single open reading frame, and two different polyadenylated 3′ UTRs. A cDNA containing an SL1 spliced leader was identified by performing PCR amplification from an early embryonic cDNA library (gift from P. Okkema) using an SL1 primer and a *sur-8*-specific primer. Full-length *sur-8* cDNA contains a 94 nucleotide SL1 spliced 5′ UTR, a 1680 nucleotide open reading frame, and either a 213 nucleotide or a 359 nucleotide 3′ UTR, depending on the polyA site used.

Molecular lesions were identified by PCR amplification of genomic DNA from lysates from one to five mutant worms and sequencing purified PCR fragments directly. For each allele, all coding regions were amplified and sequenced using primers flanking exons. The cDNA sequence differs from that predicted for AC7.1 in the positions of four splice junctions. Positions of exons and mutations corresponding to the numbering of cosmid AC7 are as follows: exon 1, 2255–2437; exon 2, 3789–3920; exon 3, 4110–4262; exon 4, 4510–4726; exon 5, 5390–5592; exon 6, 6057–6276; exon 7, 6648–6998; and exon 8, 8403–8623. *sur-8(ku167)* and *sur-8(ku242)* contained G- to A- transitions at positions 6827 and 5402, respectively.

### Two-Hybrid Strains and Plasmids

Two-hybrid reporter strains were CG1945 and Y187 (Clonetech). Strains were grown and manipulated according to the manufacturer's protocols. Two-hybrid interactions were tested by mating reporter strains transfected with expression constructs and assaying growth on $His^-$ plates followed by assaying β-galactosidase expression. *sur-8* constructs were expressed as fusion proteins with GAL4 activation domain from pACT2 (Clonetech). Full-length and mutant Ce *sur-8* were cloned by PCR from λgt11 clone #10 as NcoI-BamHI fragments into NcoI and BamHI sites of pACT2. Full-length Hs *sur-8* was PCR amplified from human brain cDNA (Clonetech) and cloned as BamHI-XhoI fragments into BamHI and XhoI sites of pACT2. pYO3 contains the full-length *lin-45 raf* cloned into pACT2 (Y. Suzuki and M. H., unpublished data). Full-length Raf1 cloned in pGAD was a gift from M. White.

*ras* constructs contained a C-to-S substitution in the CAAX box introduced by the 3′ PCR primer and were expressed as GAL4 DNA binding fusion proteins by cloning into the NdeI-BamHI sites of pAS2. Full-length *K-ras 4B*(C185S) and *N-ras*(C186S) were amplified from human brain cDNA (Clonetech). *H-ras*(V12 C186S) was cloned by amplification from *H-ras*(V12) pGSTag (a gift from K. Guan).

pMS104 and pMS105 contain *let-60 ras* and *let-60 ras*(G13E), respectively, cloned into pAS2 (M. Sundaram and M. H., unpublished data). Other *let-60 ras* mutants described were cloned by amplifying NdeI-BamHI fragments from pMH2010 (M. H., unpublished data) and cloned into pAS2.

### In Vitro Binding

Expression, purification, and nucleotide loading of GST-Ras fusion proteins was performed as described previously (Kaelin et al., 1991; Zhang et al., 1995), with the following modifications. The Glutathione Sepharose beads (Pharmacia) were washed two times with loading buffer (50 mM Tris-HCl [pH 7.5], 7.5 mM EDTA, 0.5 mg/ml bovine serum albumen [BSA], 1.0 mM DTT), then 1.0 mM GTPγS or GDPβS (Boehringer Mannheim) was added. After 1 hr of incubation at 37°C, the beads were washed two times with binding buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.0 mM DTT, 1% [v/v] Triton X-100, 5 mM MgCl2, 25 μM ZnCl2, and 0.2% [w/v] BSA).

pDS42, pDS43, pDS44, and pDS74 contain CAAX box C to S substituted *let-60 ras*, *N-ras*, *K-ras*, and *H-ras*, respectively, cloned into NdeI and BamHI sites of pGSTag (gift from K. Guan) to generate GST fusion proteins. pDS73 and pDS75 contain Hs *sur-8* and *raf1* (1–269), respectively, cloned into the BamHI and KpnI sites of pQE32 (Qiagen) to generate N-terminal 6× His-tagged fusion proteins. Fresh overnight cultures of *E. coli* DH5a transformed with pDS73 or pDS75 were diluted 1:4 in LB broth containing 100 μg/ml ampicillin and 1 mM IPTG. After 4–7 hr of growth at 37°C, pellets were collected in binding buffer containing 100 μg/ml PMSF and lysed by sonication. Lysate supernatant (2.5 μg per ml SUR-8 or 0.25 μg per ml Ras) was incubated overnight at 4°C with 15 μl of Glutathione Sepharose beads bound with GST-Ras (approximately 1 μg/ml) in a volume of 0.2 ml. Beads were washed four times with binding buffer, two times with binding buffer without BSA, and were prepared for SDS-PAGE (10%) and Western analysis. SUR-8 and Raf1 proteins were detected using an anti-5× His monoclonal antibody (Qiagen). Ras input was detected by Coomassie blue staining.

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**GenBank Accession Numbers**

The GenBank accession numbers for the *C. elegans*, human, and mouse *sur-8* cDNA and protein sequences reported in this paper are AF068919, AF068920, and AF068921, respectively.

# A PP2A regulatory subunit positively regulates Ras-mediated signaling during *Caenorhabditis elegans* vulval induction

Derek S. Sieburth,[1] Meera Sundaram,[2] Robyn M. Howard,[2] and Min Han[1,3]

[1]Howard Hughes Medical Institute, Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder, Boulder, Colorado 80309-0347 USA; [2]Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6145 USA



**We describe evidence that a regulatory B subunit of protein phosphatase 2A (PP2A) positively regulates an RTK–Ras–MAP kinase signaling cascade during *Caenorhabditis elegans* vulval induction. Although reduction of *sur-6* PP2A-B function causes few vulval induction defects in an otherwise wild-type background, *sur-6* PP2A-B mutations suppress the Multivulva phenotype of an activated *ras* mutation and enhance the Vulvaless phenotype of mutations in *lin-45 raf*, *sur-8*, or *mpk-1*. Double mutant analysis suggests that *sur-6* PP2A-B acts downstream or in parallel to *ras*, but likely upstream of *raf*, and functions with *ksr-1* in a common pathway to positively regulate Ras signaling.**

[*Key Words*: Suppressor of *ras*; phosphatase 2A; vulval induction; KSR; Raf; SUR-8]

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The protein phosphatase 2A (PP2A) enzymes, one of four families of serine/threonine protein phosphatases, are key regulators of many cellular events controlled by protein phosphorylation (Shenolikar 1994; Millward et al. 1999). PP2A is a heterotrimer, composed of a catalytic (C) subunit, an associated (A) subunit, and a regulatory (B) subunit. The C and A subunits form a core complex to which one of several classes of B subunits can bind. This differential binding of regulatory B subunits can influence the catalytic activity and substrate specificity of the catalytic core in vitro (Mayer-Jaekel and Hemmings 1994). However, although in vitro studies have identified numerous potential PP2A substrates, the broad substrate specificity has limited the ability to identify physiological targets or to understand normal PP2A regulation (Shenolikar 1994).

Recent studies have shown that PP2A can regulate signal transduction pathways. For example, PP2A stably interacts with and inactivates $Ca^{2+}$-calmodulin-dependent protein kinase IV (CaMKIV) (Westphal et al. 1998). PP2A has also been proposed to inactivate MEK and MAP kinase, because PP2A can dephosphorylate MEK and MAP kinase in vitro (for review, see Millward et al. 1999), and interference of PP2A activity by SV40 small t antigen results in activation of MAP kinase in vivo (Sontag et al. 1993). Genetic studies in *Drosophila* suggested that PP2A both positively and negatively affects Ras pathway signaling during R7 photoreceptor cell fate specification (Wassarman et al. 1996).

Here we demonstrate that a B regulatory subunit of PP2A promotes Ras signaling during *Caenorhabditis elegans* vulval development. In *C. elegans*, a *ras*-mediated signal transduction pathway in part controls the fates of six cells, the vulval precursor cells (VPCs). An inductive signal from the anchor cell activates an RTK–Ras–MAP kinase signal-transduction pathway to induce the three neighboring VPCs (P5.p, P6.p, and P7.p) to adopt vulval cell fates. These cells undergo three rounds of division followed by morphogenesis to form the vulval structure. The remaining three VPCs (P3.p, P4.p, and P8.p) adopt nonvulval cell fates and, instead, divide only once before fusing with the surrounding hypodermis (Horvitz and Sternberg 1991). Mutations that result in the mis-specification of vulval cell fates have defined many of the genes necessary for normal vulval differentiation (Kornfeld 1997; Sternberg and Han 1998). Loss-of-function mutations in positively acting components of this pathway

[3]Corresponding author.
E-MAIL mhan@colorado.edu; FAX (303) 492-7744.

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can cause fewer than three VPCs to adopt vulval cell fates, leading to a Vulvaless (Vul) phenotype. A gain-of-function mutation in *let-60 ras(n1046orG13E)*, which encodes a G13E substitution found in human Ras oncoproteins (Bos 1998), causes more than three VPCs to adopt vulval cell fates, leading to a Multivulva (Muv) phenotype.

To identify genes that act downstream of *let-60 ras*, screens have been conducted for mutations that suppress the Muv phenotype of *let-60 ras(n1046gf)* mutants. These screens have identified mutations in components acting downstream of Ras (such as *lin-45 raf*, *mek-2* MEK, and *mpk-1* MAP kinase), as well as in some new components such as *ksr-1* and *sur-8* (Sternberg and Han 1998). KSR-1 is a conserved putative kinase (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995), and SUR-8 is a conserved Ras-binding protein with leucine-rich repeats (Selfors et al. 1998; Sieburth et al. 1998). Both KSR-1 and SUR-8 appear to stimulate signaling at the level of Ras or Raf (Therrien et al. 1995; Sieburth et al. 1998; this work). Here we describe the identification and characterization of another positive regulator of Ras-mediated signaling, *sur-6*, which encodes a regulatory PR55/B subunit of PP2A (PP2A-B).

## Results

We screened for mutations that suppress the Muv phenotype caused by an activated *let-60 ras* mutation, *n1046gf* (Sundaram and Han 1995) or that enhance the Vul phenotype caused by a hypomorphic *lin-45 raf* mutation, *ku112* (M. Sundaram, unpubl.). These screens identified two mutations, *ku123* and *cs24*, that define the gene *sur-6* (suppressor of ras). These *sur-6* mutations cause few or no vulval defects in an otherwise wild-type background; *ku123* and *cs24* mutants display an average vulval induction of 100% or 99%, respectively (Table 1). However, both *sur-6* mutations suppress the Muv phenotype of *let-60(n1046gf)* animals (Table 2), and enhance the vulval induction defects and larval lethality caused by weak alleles of *lin-45 raf* (Table 1). These strong genetic interactions suggest that the sur-6 mutations reduce signaling by the Ras pathway at a point downstream or in parallel to *let-60 ras*. *sur-6(ku123)* and *sur-6(cs24)* appear to strongly reduce (but not eliminate) *sur-6* gene function. These *sur-6* alleles and a deficiency of the *sur-6* locus each semidominantly suppress the Muv phenotype of *let-60(n1046gf)* mutants (Table 2), suggesting the *sur-6* locus is haplo insufficient. RNA-mediated inhibition of *sur-6* also suppresses the *let-60(n1046gf)* Muv phenotype and causes a partial Vul phenotype in a wild-type background (Table 3), arguing that these are loss-of-function phenotypes. *qDf8* fails to complement the weak Vul phenotype of *sur-6(cs24)* mutants, and *sur-6/sur-6* homozygotes and *sur-6/qDf8* hemizygotes display similar phenotypes (Tables 1 and 2), consistent with the *sur-6* mutations strongly reducing sur-6 function. This notion is further supported by the fact that the suppressor phenotype of *sur-6(ku123)* can be rescued by injecting wild-type DNA containing the *sur-6* gene (see below and Materials and Methods). Nevertheless, the *sur-6* mutations are likely non-null, because RNA inhibition suggests that the *sur-6* null phenotype is embryonic lethal (Table 3; see below). Because reducing sur-6 function reduces vulval induction in sensitized genetic backgrounds, we conclude that *sur-6* normally plays a positive role in regulating Ras pathway signaling during vulval development.

*sur-8* and *ksr-1* are two other genes that display genetic interactions similar to those of *sur-6*. Strong loss-of-function mutations in *sur-8* or *ksr-1* cause few defects on their own, but strongly modify the phenotypes of other Ras pathway mutants (Kornfeld et al. 1995; Sundaram and Han 1995; Sieburth et al. 1998). We found that *sur-6;sur-8* double mutants display a synthetic Vul phenotype (Table 1), consistent with these mutations potentially affecting different aspects of Ras pathway regulation. Interestingly, however, *sur-6;ksr-1* double mutants resemble the *sur-6* or *ksr-1* single mutants (Table 1). Because reducing the function of either *ksr-1* or *sur-6* has the same effect as reducing the function of both, it is likely that *sur-6* acts together with *ksr-1* in a common signaling pathway to regulate Ras signaling.

*raf* genetically acts downstream of *ras* in *C. elegans* and in *Drosophila* (Dickson et al. 1992; Han et al. 1993), and mammalian Raf is a direct Ras effector (for review, see Katz and McCormick 1997). *Drosophila ksr* has been shown previously to function genetically downstream or in parallel to *ras* and upstream of *raf* (Therrien et al. 1995). We tested whether this was also true for *C. elegans ksr-1* and *sur-6*. An activated *raf* transgene under the control of a heat shock promoter [*HSP–raf(gf)*] causes a Muv phenotype that can be suppressed by mutations in the downstream Ras pathway components *mek-2* MEK or *mpk-1* MAP kinase (Sieburth et al. 1998). In contrast, this *HSP–raf(gf)* Muv phenotype is not suppressed by *ksr-1* or *sur-6* mutations (Table 2). Therefore, *ksr-1* and *sur-6* likely act upstream of *lin-45 raf*. Consistent with this, *sur-6(ku123)* also fails to suppress the Muv phenotype caused by a relatively weak mutation of *lin-1*, which encodes an ETS domain transcription factor acting downstream of *mpk-1* MAP kinase (Jacobs et al. 1998; Tan et al. 1998). The genetic placement of *ksr-1* and *sur-6* suggests that *ksr-1* and *sur-6* may act in a regulatory branch that modifies or cooperates with Ras/Raf.

We cloned *sur-6* by genetic mapping followed by transformation rescue (see Materials and Methods). An 11-kb fragment, pDS89, which contains *sur-6(+)* rescuing activity was predicted by the *C. elegans* genome sequencing consortium to contain a single gene (F26E4.1) that encodes a PR55 family regulatory B subunit of PP2A (PP2A-B, Fig. 1). The *sur-6* alleles *ku123* and *cs24* each contain single G → A substitutions that introduce amino acid substitutions at highly conserved positions within PP2A-B (Fig. 1). The PP2A-B coding region, when expressed under the control of a heat shock promoter in transgenic animals, can rescue the *sur-6(ku123)* mutant (see Materials and Methods), and RNA interference of PP2A-B phenocopies the *sur-6* suppression and partial Vul phenotypes (Table 3). Thus, we conclude that *sur-6*

**Table 1.** *sur-6 mutations enhance the vulval defects of* lin-45 *Raf,* mpk-1 *MAP kinase, or* sur-8 *but not* ksr-1 *mutants*

| Genotype[a] | Percent Average Vul[b] | Percent Average induction[c] | (n) | Percent larval lethal[d] (n) |
|---|---|---|---|---|
| *sur-6(ku123)* | 0 | 100 | (26) | 0 (474) |
| *sur-6(ku123)/qDf8* | 0 | 100 | (28) | 0 (531)[e] |
| *sur-6(cs24)* | 2 | 99 | (48) | 0 (195) |
| *sur-6(cs24)/qDf8* | 8 | 97 | (26) | 0 (386)[e] |
| *+/qDf8* | 0 | 100 | (16) | N.D. |
| *cs24/+* | 0 | 100 | (35) | N.D. |
| *lin-45(sy96)* | 58 | 53 | (38) | 86 (290) |
| *sur-6(ku123); lin-45(sy96)* | 100 | 5 | (17) | 94 (228) |
| *lin-45(ku112)* | 0 | 100 | (26) | <1 (66) |
| *sur-6(cs24); lin-45(ku112)* | 87 | 50 | (24) | 80 (360) |
| *mpk-1(ku1)* | 17 | 97 | (29) | 7 (229) |
| *sur-6(ku123); mpk-1(ku1)* | 82 | 76 | (28) | 77 (263) |
| *sur-8(ku167)* | 0 | 100 | (18) | <1 (271) |
| *sur-6(ku123); sur-8(ku167)*[f] | 71 | 56 | (24) | <1 (263) |
| *sur-6(cs24); sur-8(ku167)* | 65 | 67 | (20) | 3 (198) |
| *ksr-1(ku68)* | 0 | 100 | (23) | 24 (257) |
| *sur-6(ku123); ksr-1(ku68)* | 3 | 99 | (40) | 17 (282) |
| *sur-8(ku167); ksr-1(ku68)*[g] | 100 | 4 | (19) | 85 (164) |
| *ksr-1(n2526)* | 1 | 99 | (68) | 2 (607) |
| *sur-6(cs24); ksr-1(n2526)* | 3 | 99 | (62) | 1 (88) |
| *sur-8(ku167); ksr-1(n2526)* | 67 | 64 | (24) | 54 (132) |

[a] *ksr-1(ku68)* (R531H) is a strong loss-of-function, possibly dominant-negative allele (Sundaram and Han 1995). *ksr-1(n2526)* (W255stop) is a putative null allele (Kornfeld et al. 1995). *lin-45(sy96)* and *lin-45(ku112)* are hypomorphic (partial loss-of-function) alleles (Han et al. 1993; Sundaram and Han 1995); *lin-45(sy96)* contains a splice site mutation, and the *lin-45(ku112)* lesion is unknown. *sur-8(ku167)* (E430K) is a strong hypomorphic allele (Sieburth et al. 1998). *mpk-1(ku1)* (A38V) is a hypomorphic allele (Wu and Han 1994). *qDf8* removes the flanking genes *mec-8* and *fog-3* and thus should remove *sur-6* (R. Ellis, pers. comm.). For deficiency strains, *sur-6* was marked with *unc-13*, and *qDf8* was marked with *ces-1*. No other defects were observed in *sur-6/qDf8* strains. *lin-45(sy96)* was linked to *dpy-20*, *ksr-1(ku68)* was linked to *lon-2*. *lin-45(ku112)* was linked to *dpy-20* for double mutants with *ksr-1*. *sur-6(ku123)* was linked to *unc-29* for double mutants with *lin-45*, *ksr-1*, and *sur-8*. All other strains were unmarked. The *dpy-20*, *unc-29*, and *lon-2* markers were also tested in the control strains to show that they have no effects on vulval induction.

[b] Percent of animals in which <3 VPCs adopted vulval fates, as scored under Nomarski optics (Sieburth et al. 1998). In some *sur-6* strains, VPCs were occasionally undivided or absent and may have adopted 4° fates (Clark et al. 1993). Nondivision of VPC P5.p, P6.p, or P7.p occurs infrequently (4/24 *ku123*; *ku167* animals, 3/23 *cs24,ku167* animals, 3/65 *cs24,n2526* animals). %Vul is calculated only for those animals in which P5.p, P6.p, and P7.p were present and divided at least once.

[c] Average percent of VPCs adopting a vulval cell fate, as scored under Nomarski optics (Sieburth et al. 1998; 100% for wild type). Induction is calculated only for those animals in which P5.p, P6.p, and P7.p were present and divided at least once.

[d] Percent of animals arresting in early larval stages with a clear, rod-like phenotype (Sieburth et al. 1998). (N.D.) Not determined.

[e] For *qDf8* experiments, *n* corresponds to the entire brood of *sur-6/qDf8* mothers, half of which should also have been *sur-6/qDf8* hemizygotes. No more than 25% of such broods arrested as embryos, suggesting that *sur-6/qDf8* (like *sur-6/sur-6*) has no significant embryonic lethal phenotype.

[f] *sur-6(ku123); sur-8* double mutants were 37% embryonic lethal with an average brood size (live worms plus dead embryos) of 26 (*n* = 263).

[g] Data from Sieburth et al. (1998).

encodes PP2A-B. SUR-6 shares >59% overall amino acid identity with PP2A-B from human or *Drosophila*, with three large stretches of at least 75% identity (Fig. 1B). In mammals there are three PR55/B isoforms that differ in spatial and temporal expression (Mayer-Jaekel and Hemmings 1994). SUR-6 is most similar to the mammalian Bα subtype.

PP2A-B subunits modulate the activity and/or substrate specificity of the PP2A-A/C catalytic core (Mayer-Jaekel and Hemmings 1994). SUR-6 is the only predicted PR55/B family member encoded by the *C. elegans* genome, although other types of regulatory B subunits (such as PR56/B′) are also present. The *C. elegans* genome is predicted to encode a single PP2A-A subunit (F48E8.5) and a single PP2A-C subunit (F38H4.9), which share >90% amino acid identity with their mammalian counterparts. As expected, the PP2A-A subunit can bind to both SUR-6 PP2A-B and the PP2A-C subunit, as assayed by the yeast two-hybrid system (data not shown). Given the positive role of *sur-6* PP2A-B defined by genetic analysis, *sur-6* PP2A-B could either function to activate the catalytic core, which in turn would activate Ras pathway signaling, or it could function to relieve inhibition of Ras signaling by the core complex (Fig. 2A).

To determine the requirements for PP2A during *C. elegans* development, we used RNA interference (RNAi) (Fire et al. 1998) to block *sur-6* PP2A-B, PP2A-A, or PP2A-C expression. For each PP2A gene (but not for

**Table 2.** *Epistasis analysis of* sur-6 *and* ksr-1 *with Muv mutations*

| *sur-6* or *ksr-1* mutation[a] | Muv mutation[b] | Percent Muv[c] (*n*) | Percent average induction[d] (*n*) |
|---|---|---|---|
| +/+ | +/+ | 0 (many) | 100 (many) |
| +/+ | *let-60(n1046gf)* | 87 (276) | 154 (27) |
| *sur-6(ku123)* | *let-60(n1046gf)* | 6 (240) | 103 (26) |
| *sur-6(cs24)* | *let-60(n1046gf)* | 19 (107) | 104 (33) |
| *sur-6(ku123)/ sur-6(cs24)* | *let-60(n1046gf)* | 3 (31) | N.D. |
| *sur-6(ku123)/+* | *let-60(n1046gf)* | 22 (148) | 115 (31) |
| *sur-6(cs24)/+* | *let-60(n1046gf)* | 40 (53) | N.D. |
| *sur-6(ku123)/ qDf8* | *let-60(n1046gf)* | 1 (209) | N.D. |
| *+/qDf8* | *let-60(n1046gf)* | 5 (281) | N.D. |
| +/+ | *HSP–raf(gf)* | 47 (30) | 119 (30) |
| *sur-6(ku123)* | *HSP–raf(gf)* | 47 (17) | 125 (17) |
| +/+ | *HSP–raf(gf)* | 47 (17) | 118 (17) |
| *ksr-1(ku68)* | *HSP–raf(gf)* | 46 (28) | 117 (28) |
| +/+ | *lin-1(e1275)* | 99 (68) | N.D. |
| *sur-6(ku123)* | *lin-1(e1275)* | 100 (102) | N.D. |
| +/+ | *lin-15(n765)* | 100 (59) | 157 (28) |
| *sur-6(ku123)* | *lin-15(n765)* | 6 (351) | 102 (20) |

[a]*ku123* was marked with unc-29 in all strains except those with *n1046*. The full genotype of *ku123/cs24* was *n1046/sy130 dpy-20; ku123/cs24*. The full genotype of *ku123/+* was *ku123/unc-29; n1046; him-5/+*. The full genotype for *cs24/+* was *n1046/ sy130 dpy-20; cs24/+*. *sy130* encodes the same *let-60 ras(G13E)* substitution as *n1046*. Full genotypes for deficiency analysis: *ces-1 qDf8/unc-13 ku123; n1046* and *ces1 qDf8/ unc-13 dyp-24; n1046. HSP–raf(gf); ksr-1(ku68)* animals (and their paired controls) were marked with unc-24.

[b]For *HSP–raf(gf)* description, see text and Sieburth et al. (1998). Also see Sieburth et al. (1998) for positive control results (suppression of *HSP–raf(gf)* by *mek-2* and *mpk-1* alleles). *HSP–raf(gf)* strains were heat shocked during the early-mid L3 stage for 80 min at 36°C. *lin-15* strains were grown at 18°C.

[c]Percent Muv was determined by examining adult hermaphrodites with a dissecting microscope for the presence of ectopic ventral protrusions [for *let-60(gf)*, *lin-1* and *lin-15* experiments], or by examining L4 larvae under Nomarski optics [for *HSP–raf(gf)* experiments].

[d]See Table 1 footnote. In 1/34 *cs24; n1046gf* animals, P4.p and P8.p were undivided and may have adopted 4° fates. Induction is calculated here only for animals in which P(4-8).p divided at least once.

*sur-8* or *ksr-1*), RNAi caused highly penetrant embryonic lethality in both wild-type and *let-60(n1046gf)* backgrounds. Embryos arrested at ~100 cell stage, with widely variable cell sizes (data not shown). Thus, unlike *ksr-1* and *sur-8*, PP2A appears to be absolutely required during embryonic development in addition to functioning later during vulval induction. Because the *sur-6(ku123)* and *sur-6(cs24)* mutations caused little or no embryonic lethality, even when hemizygous (Table 2), the two functions of *sur-6* PP2A-B appear separable, with these *sur-6* point mutations primarily affecting *sur-6* PP2A-B function in vulval development but not in embryogenesis.

To avoid the PP2A(RNAi) lethality and test its effects on vulval development, we examined the last surviving progeny of RNA-injected mothers (Table 3). In this assay, *sur-6(RNAi)* caused a partial Vul phenotype in a wild-type background and efficiently suppressed the *let-60(n1046gf)* Muv phenotype. However, PP2A-C(RNAi) caused few vulval defects and only weakly suppressed the *let-60(n1046gf)* Muv phenotype. Similar results were obtained by use of a hypodermal-specific promoter to drive expression of *sur-6* PP2A-B or PP2A-C RNAs (Table 3). Thus, it is still unclear whether PP2A catalytic activity promotes and/or inhibits Ras-mediated vulval induction.

## Discussion

We have shown for the first time a positive and specific

**Table 3.** *RNA interference of* sur-6 *PP2A-B, PP2A-C, or PP2A-A*

| Genotype | dsRNA[a] or transgenes[b] | Vul or Muv (*n*) | Percent avg. induction (*n*) |
|---|---|---|---|
| + | no dsRNA | 0 (many) | 100 (many) |
| + | *sur-6* PP2A-B | 19 Vul (21) | 94 (21) |
| + | PP2A-C | 5 Vul (22) | 99 (22) |
| *let-60(n1046gf)* | no dsRNA | 95 Muv (19) | 173 (19) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 3 Muv (29) | 101 (29) |
| *let-60(n1046gf)* | PP2A-C | 70 Muv (23) | 132 (23) |
| *let-60(n1046gf)* | *sur-8* | 15 Muv (33) | 106 (33) |
| *let-60(n1046gf)* | no dsRNA | 93 Muv (59) | 143 (59) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 7 Muv (15) | 103 (15) |
| *let-60(n1046gf)* | PP2A-A | 57 Muv (14) | 125 (14) |
| *let-60(n1046gf)* | no transgenes | 82 Muv (242) | 175 (23) |
| *let-60(n1046gf)* | *sur-6* PP2A-B | 20 Muv (172) | 110 (48) |
| *let-60(n1046gf)* | PP2A-C | 65 Muv (82) | N.D. |

[a]In the case of *sur-6* PP2A-B, PP2A-C or PP2A-A (but not *sur-8*), nearly complete embryonic lethality was observed in progeny laid 8–24 hr after double-stranded (ds) RNA injection. Most surviving *sur-6(RNAi)* animals displayed a weakly Uncoordinated phenotype. Vulval induction was scored in surviving progeny laid at least 6 hr postinjection (for the wild-type background), or in progeny laid 6–8 hr postinjection, before the lethal period began [for the *let-60(gf)* background]. In the wild-type background, *sur-6 (RNAi)* caused variable vulval defects, including defects in vulval induction (2° to 3° or hybrid fate transformation; 5/31 animals), VPC generation (absence of P5.p, P6.p, or P7.p, or failure of those cells to divide; 10/31 animals), and vulval lineage execution (failure of P5.pxx to complete last round of vulval division; 1/31 animals). The VPC generation defects were almost never seen in the *let-60(n1046gf)* background. In the wild-type background, PP2A-C RNAi also caused occasional defects in vulval induction (P5.p executed a "hybrid" lineage, generating only 3 vulval descendants and 1 nonvulval descendant; 1/23 animals), VPC generation (absence of P5.p; 1/23 animals), and vulval lineage execution (failure of P5.pxx to complete last round of vulval division; 1/23 animals). %Vul (and induction) are calculated only for animals in which P5.p, P6.p, and P7.p were present and divided at least once. %Muv (and induction) are calculated only for animals in which P(4-8).p were present and divided at least once.

[b]We used the *col-10* promoter to coexpress both sense and antisense RNA fragments in the hypodermis (see Material and Methods).

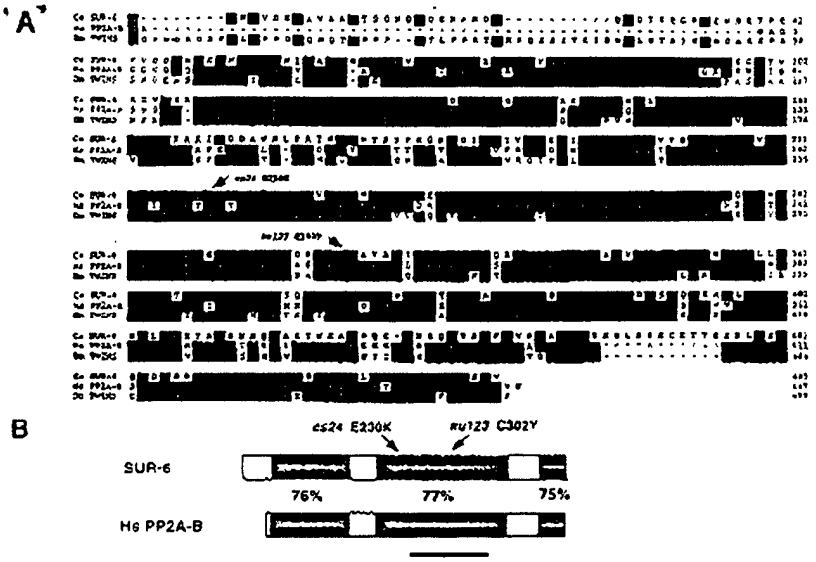


Figure 1. Sequence comparison between SUR-6 PP2AB and human PP2A-Bα. (*A*) Amino acid alignment of *C. elegans* SUR-6 PP2A-B (as predicted by cDNA analyses; see Materials and Methods, GenBank accession no. AF174643) with a human PP2A-Bα isoform (GenBank accession no. 231445; Mayer et al. 1991) and *Drosophila Twins* PP2A-B (GenBank accession no. S43716; Uemura et al. 1993). Identical amino acids are shaded. The position of the C302Y and E230K missense mutations identified in *sur-6(ku123)* and *sur-6(cs24)* mutants, respectively, are denoted. C302 and E230 are conserved in PP2A-B from all species in which it has been identified, from human to yeast. (*B*) Structural comparison of *sur-6* PP2A-B and human PP2A-Bα non-neuronal isoform. Percent amino acid identity is shown for shaded regions. Nonshaded regions share 20% or less amino acid identity. The overall amino acid identity is 59%. The bar denotes the region with similarity to the c-Abl proposed substrate-binding domain (amino acids 257–378).

regulatory role for a PP2A regulatory PR55/B subunit in Ras-mediated signal transduction. We propose that *sur-6* PP2A-B influences the catalytic activity of PP2A toward specific Ras pathway substrate(s), such as Raf or KSR, leading to the enhancement of Ras pathway signaling.

Genetic analysis shows that *sur-6* PP2A-B positively regulates Ras pathway signaling during vulval induction. First, reducing *sur-6* PP2A-B function by mutation or RNA inhibition suppresses the excess vulval cell fate specification caused by an activated *let-60 ras* allele (but not that caused by an activated *raf* transgene). Second, *sur-6* PP2A-B mutations enhance defects in vulval cell-fate specification caused by weak mutations in the Ras pathway components *lin-45 raf* or *mpk-1* MAP kinase. However, reducing *sur-6* PP2A-B function by mutation or RNA inhibition only mildly reduces vulval induction in an otherwise wild-type background, suggesting that *sur-6* PP2A-B activity might not be essential for vulval induction when other components are functioning normally.

*sur-6* PP2A-B appears to regulate Ras pathway signaling in multiple tissues besides vulval precursor cells. For example, a *sur-6* PP2A-B mutation suppresses the male mating defect caused by an activated *ras* mutation (data not shown) and enhances the rod-like larval lethality of *lin-45 raf* or *mpk-1* MAP kinase mutants. However, *sur-6* PP2A-B also appears to have functions that are currently not known to involve the Ras pathway. RNA inhibition of *sur-6* PP2A-B function reveals an absolute requirement for *sur-6* during embryogenesis. Additional roles of *sur-6* PP2A-B in the generation or survival of VPCs, and in the development or function of muscles and/or motor neurons, are suggested by the fact that *sur-6(cs24)* animals and surviving *sur-6(RNAi)* animals sometimes lack one or more VPCs and are weakly uncoordinated. It would be interesting to know whether or not these defects also involve the Ras pathway.

SUR-6 PP2A-B likely functions by regulating the activity of the PP2A-A/C core complex. Mammalian PP2A-B regulatory subunits can either activate or inhibit core activity in vitro, depending on the substrate used (Mayer-Jaekel and Hemmings 1994). Genetic data in yeast support specific activating roles for PP2A regulatory subunits, because mutations in *cdc55* (a PR55/B subunit) and *rts1* (a PR56/B′ subunit) each cause a distinct subset of phenotypes associated with mutations in the PP2A catalytic core (Shu et al. 1997). If SUR-6 PP2A-B also functions to activate the PP2A catalytic core, this implies that PP2A positively regulates Ras signaling at the level of Ras or Raf (Fig. 2). Such a model would differ from previously proposed models, on the basis of experiments overexpressing SV40 small t antigen in cultured mammalian cells, that suggest PP2A inhibits MEK and MAP kinase activities (Sontag et al. 1993). However, it is likely that regulation of the Ras pathway by PP2A-C is complex, involving multiple positive and negative influences on different substrates (e.g., Wassarman et al. 1996). *sur-6* PP2A-B may stimulate PP2A core activity toward a specific subset of these substrates, such as KSR and/or Raf.

Consistent with the idea that sur-6 PP2A-B acts by activating a PP2A core is the observation that the *sur-6(ku123)* allele affects an absolutely conserved cysteine residue within a region of PP2A-B that shares similarity to c-Abl kinase domains VI–X. This region of similarity is shared by nonkinase residues of c-Abl that may be involved in target specificity, and may thus define a region of PP2A-B involved in localization or target specificity (Mayer et al. 1991). We hypothesize that in *sur-6(ku123)* PP2A-B mutants, the PP2A core may be mislocalized or may fail to be targeted to the proper substrates. This model would be similar to the suggestion from a recent work in mammalian cells and *Xenopus* embryo explants that a PR56/B′ subunit of PP2A may interact with APC and direct PP2A to dephosphorylate specific

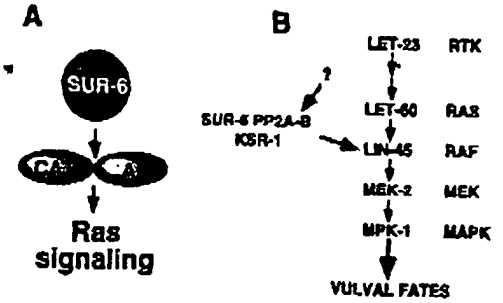


Figure 2. Models for SUR-6 PP2A-B function in the vulval induction pathway. (*A*) SUR-6 PP2A-B is likely to regulate the activity of a PP2A core complex composed of a C and A subunit to influence Ras signaling. SUR-6 PP2A-B is a positive regulator of Ras signaling, so SUR-6 could function either to activate the PP2A core, which in turn activates Ras signaling, or to inhibit the PP2A core that normally inhibits Ras signaling. Potential targets for SUR-6 PP2A-B-regulated PP2A activity include LIN-45 Raf and KSR-1. (*B*) Model for the signal transduction pathway regulated by SUR-6. The receptor tyrosine kinase LET-23 is activated by an inductive signal that leads to the activation of LET-60 Ras. Ras–GTP recruits Raf kinase to the membrane and Raf phosphorylates and activates the MEK-2 MEK/MPK-1 MAPK kinase cascade. We showed that SUR-6 PP2A-B positively regulates signaling at a branch point at the level of Ras or Raf. KSR-1 and SUR-6 may work together to strengthen Ras pathway output during vulval cell fate specification.

components of the Wnt/β-catenin signaling pathway (Seeling et al. 1999).

Interestingly, our data suggest that *sur-6* PP2A-B may function together with *ksr-1*. *sur-6* PP2A-B and *ksr-1* have the same epistatic relationship with respect to *ras* and *raf*, and *sur-6; ksr-1* double mutants resemble *sur-6* or *ksr-1* single mutants. Taken together, these data are consistent with the idea that *sur-6* PP2A-B and *ksr-1* act in a common pathway to stimulate *ras*-mediated signaling at a branch point that feeds out of the pathway at the level of *ras* or into the pathway at the level of *raf* (Fig. 2B).

KSR proteins positively regulate Ras signaling in *C. elegans* and *Drosophila* (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995), as well as in *Xenopus* oocytes and certain mammalian cells (Therrien et al. 1996). Murine KSR associates with several proteins in vivo, including Raf, MEK, and MAP kinase, and has been proposed to function as a scaffold protein involved in signal propagation through the Raf/MEK/MAP kinase cascade (Therrien et al. 1996; Stewart et al. 1999). Murine KSR is a phosphoprotein (Cacace et al. 1999); although the role of phosphorylation in KSR regulation is unclear. Thus, KSR-1 is a potential target for regulation by PP2A during vulval induction. Alternatively, KSR-1 may act to regulate PP2A-B function.

Another potential *sur-6* PP2A-B-dependent PP2A target is LIN-45 Raf. The mechanism of Raf activation is still poorly understood, but there is evidence for both inhibitory and activating phosphates on Raf (Morrison and Cutler 1997). Whereas in vitro studies suggest that PP2A can dephosphorylate Raf, it is probably not the major phosphatase to remove activating phosphates (Dent et al. 1995). However, a role for PP2A in removing inhibitory phosphates has not been ruled out. The placement of a B regulatory subunit of PP2A as a positive regulator of the Ras pathway, and the unexpected finding that it acts together with KSR-1, should lead to a better understanding of PP2A regulation and its physiological substrates.

## Materials and methods

Mutants were derived from the wild-type Bristol strain, N2, and grown under standard conditions (Brenner 1974) at 20°C unless otherwise indicated. Some strains were obtained from the *Caenorhabditis* Genetics Center. The alleles and deficiencies used are described in Riddle et al. (1997) unless otherwise indicated: LGI, *unc-29(e1072, h1), ces-1(n703), unc-13(e1091), dpy-24(s71), qDf8, qDf5*; LGIII, *mpk-1(ku1), unc-119(ed3)*; LGIV, *lin-1(e1275), sur-8(ku167)* (Sieburth et al. 1998), *unc-24(e138), lin-45(sy96), lin-45(ku112)* (Sundaram and Han 1995), *let-60(n1046gf), let-60(sy130gf), dpy-20(e1282)*; LGV, *him-5(e1490)*; LGX, *ksr-1(ku68), ksr-1(n2526), lin-15(n765)*.

### *sur-6* isolation and cloning

*sur-6(ku123)* was isolated as a dominant suppressor in screens for suppressors of the Muv phenotype of *let-60 ras(n1046gf)* homozygotes described previously (Wu and Han 1994; Sundaram and Han 1995). *sur-6(cs24)* was isolated in screens for enhancers of the *lin-45 raf(ku112)* Vul and lethal phenotypes (M. Sundaram, unpubl.). Both alleles were obtained after ethylmethanesulfonate mutagenesis.

*sur-6* was first mapped between *unc-29* and *dpy-24* of linkage group I by standard three-point mapping. *sur-6* was further mapped with *unc-29(h1) hP6 dpy-24; let-60(n1046)*, derived from SP1726 (gift from E. Lundquist, University of Minnesota, St. Paul, MN). Unc non-Dpy recombinants were tested for *ku123* by scoring the suppression phenotype and for the polymorphism *hP6* by PCR with primers Tc1-1 and hP6-B (gift from D. Fitch, New York University, NY, NY). A total of 15 of 35 Unc-29 recombinants contained *ku123* and of these, one was positive for *hP6*, placing *sur-6* to the right of cosmid C03D6. *sur-6* mapped to the left of cosmid F14G10, which is the left endpoint of the complementing deficiency *qDf5* (R. Ellis, pers. comm.). Deficiency *qDf8* uncovers *sur-6* (Tables 1 and 2). *sur-6(cs24)* was mapped to an interval between *dpy-5* and *unc-101*, and within two map units of *unc-13* by standard mapping crosses.

Cosmids in the region were tested for *sur-6(+)* activity by assaying their ability to rescue the *sur-6(ku123)* suppressor phenotype. Cosmids were injected at 5–10 μg/ml together with 40 μg/ml *unc-119(+)* transformation marker pDP#MM016 (Maduro and Pilgrim 1995) and 10 μg/ml pBluescript into *sur-6(ku123); unc-119; let-60* mutants. A single cosmid, K02A11 contained *sur-6(+)* rescuing activity, but an overlapping cosmid, F26E4, failed to rescue.

Northern analysis of mixed-stage RNA with a genomic fragment that spanned all predicted exons of F26E4.1 as a probe revealed the presence of an abundant 2.5-kb transcript and two minor transcripts of 2.3 and 1.8 kb. Approximately 1 million plaques were screened from a λgt11-mixed stage cDNA library (gift from P. Okkema, University of Illinois, Chicago, IL) with a 1.5-kb genomic probe, and 22 positive clones were isolated. One positive clone contained a partial SL1-spliced leader correspond-

ing to base pair 19414 of cosmid K02A11. Positive clones analyzed differed in the length of the 3′ UTR because of differential use of transcription termination sites. The sequence of these clones confirmed the exon/intron structure predicted by the *C. elegans* genome sequencing consortium. A transgene (pDS54) that expresses the *sur-6* PP2A-B cDNA under the control of the Hsp16-41 promoter could rescue the suppressor phenotype of *sur-6(ku123)*. The *sur-6(ku123); let-60(n1046gf)* double mutant was used to host the transgene. When heat-shocked, 75% of the transgenic animals were Muv (average vulval induction is 138%, $n = 12$), compared with 0% Muv (100% vulval induction, $n = 20$) for non-heat-shocked controls.

The molecular lesion associated with *sur-6* mutations was identified by PCR amplifying genomic DNA from lysates of *sur-6* mutants and sequencing PCR products directly.

### *RNAi*

RNAi with double-stranded RNA was performed essentially as described (Fire et al. 1998). PCR fragments containing >1 kb of the coding regions were used as templates for in vitro transcription reactions. RNA was injected in parallel into either *let-60(n1046gf)* or N2 hermaphrodites at a concentration of 0.5–1 mg/mL.

The *col-10* promoter was used to coexpress both sense and antisense RNA fragments in hypodermis. The *sur-6* PP2A-B transgenes are pDS94 and pDS95, which contain nucleotides 90-1488 of *sur-6* cloned in opposite orientations into a *col-10* promoter-containing vector. The PP2A-C transgenes are pDS96 and pDS97, which contain nucleotides 241-957 of PP2A-C cloned in opposite orientations into the *col-10* promoter vector. Transgenes were coinjected with the marker pTG96 (Yochem et al. 1998).

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